

Characterization of a Novel Endocannabinoid, Virodhamine, with Antagonist Activity at the CB1 Receptor

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ABSTRACT

The first endocannabinoid, anandamide, was discovered in 1992. Since then, two other endocannabinoid agonists have been identified, 2-arachidonoyl glycerol and, more recently, noladin ether. Here, we report the identification and pharmacological characterization of a novel endocannabinoid, virodhamine, with antagonist properties at the CB1 cannabinoid receptor. Virodhamine is arachidonic acid and ethanolamine joined by an ester linkage. Concentrations of virodhamine measured by liquid chromatography atmospheric pressure chemical ionization-tandem mass spectrometry in rat brain and human hippocampus were similar to anandamide. In peripheral tissues that express the CB2 cannabinoid receptor, vi-

rodhamine concentrations were 2- to 9-fold higher than anandamide. In contrast to previously described endocannabinoids, virodhamine was a partial agonist with in vivo antagonist activity at the CB1 receptor. However, at the CB2 receptor, virodhamine acted as a full agonist. Transport of [14 C]anandamide by RBL-2H3 cells was inhibited by virodhamine. Virodhamine produced hypothermia in the mouse and acted as an antagonist in the presence of anandamide both in vivo and in vitro. As a potential endogenous antagonist at the CB1 receptor, virodhamine adds a new form of regulation to the endocannabinoid system.

Following the discovery of two G protein-coupled receptors, CB1 and CB2, which respond to Δ^9 -tetrahydrocannabinol, the active principal in marijuana, a search was initiated to identify endogenous ligands for these receptors. Anandamide (*N*-arachidonoyl ethanolamide) was the first endocannabinoid discovered in 1992 by screening porcine brain extracts for compounds that bound to the cannabinoid receptor (Devane et al., 1992). It was later shown that anandamide could stimulate cannabinoid receptor-mediated signal transduction (Felder et al., 1993). The second endocannabinoid identified was 2-arachidonoyl glycerol (2-AG), which was isolated from canine gut and shown to have in vivo effects similar to Δ^9 -tetrahydrocannabinol (Mechoulam et al., 1995). Very recently, a third endocannabinoid, noladin ether, was also isolated from porcine brain (Hanus et al., 2001). Both anandamide and 2-AG are agonists at both the CB1 and CB2 receptors. Noladin ether has been shown to bind to the CB1 receptor with nanomolar affinity and to the CB2 receptor with low micromolar affinity, but functional activity has not yet been determined (Hanus et al., 2001).

In the course of development of a bioanalytical method to measure anandamide in brain and peripheral tissues and

brain microdialysate, a second analyte was seen that had the same molecular weight as anandamide but a shorter retention time, and therefore, could not be anandamide (Fig. 1). The peak was hypothesized to be *O*-arachidonoyl ethanolamine, and an authentic standard was subsequently synthesized (BIOMOL Research Laboratories, Plymouth Meeting, PA). Based on its chromatographic and mass spectrometric properties compared with the synthesized standard, the unknown analyte was confirmed to be *O*-arachidonoyl ethanolamine. *O*-Arachidonoyl ethanolamine is arachidonic acid and ethanolamine joined by an ester linkage, the opposite of the amide linkage found in anandamide. Based on this opposite orientation, the molecule was named virodhamine from the Sanskrit word *virodha*, which means opposition.

Synthetic virodhamine was used to measure and characterize its in vitro and in vivo function. Tissue concentrations were measured in rat and human brain as well as rat peripheral tissues and rat striatal microdialysate and compared with anandamide. The in vitro functional activity of virodhamine at CB1 and CB2 receptors was measured by antibody capture GTP binding. The ability of virodhamine to decrease body temperature in mouse, a property of CB1 cannabinoid receptor

ABBREVIATIONS: Anandamide, *N*-arachidonoyl ethanolamide; 2-AG, 2-arachidonoyl glycerol; LC, liquid chromatography; APCI-MS-MS, atmospheric pressure chemical ionization-tandem mass spectrometry; DMSO, dimethyl sulfoxide; [35 S]GTP γ S, guanosine 5'-*O*-(3-[35 S]thio)triphosphate; methanandamide, *R*(+)-arachidonoyl-1'-hydroxy-2'-propylamide; AM404, *N*-(4-hydroxyphenyl)arachidonylamide; WIN 55,212-2, *R*(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone.

ligands, was characterized *in vivo*. Together, these data indicate that virodhamine acts as a unique endogenous cannabinoid ligand with antagonist activity at the CB1 receptor.

Materials and Methods

Drugs. WIN 55,212-2 [*R*-[+]-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone] was purchased from Tocris Cookson (St. Louis, MO). AM404 [*N*-(4-hydroxyphenyl)arachidonamide], anandamide, and methanandamide [*R*(+)-arachidonyl-1'-hydroxy-2'-propylamide] were purchased from Sigma/RBI (Natick, MA). 2-AG was purchased from BIOMOL Research Laboratories. Virodhamine (*O*-arachidonoyl ethanolamine) and [$^2\text{H}_8$]anandamide were custom synthesized by BIOMOL Research Laboratories.

Liquid Chromatography (LC) Atmospheric Pressure Chemical Ionization-Tandem Mass Spectrometry (APCI-MS-MS) Analysis. A quantitative bioanalytical method utilizing LC APCI-MS-MS was used to measure anandamide and virodhamine. Following liquid extraction with organic solvent and further purification by solid phase extraction, samples were separated using a Zorbax Eclipse XDB C-18 column (75 × 4.6 mm, 3.5 μm, 80 Å; Phenomenex, Torrance, CA) under isocratic conditions with a 90:10, methanol/5 mM ammonium acetate, 0.25% glacial acetic acid mobile phase at a flow rate of 1 ml/min. Quantification of anandamide and virodhamine was accomplished using *m/z* 348 ($[\text{M} + \text{H}]^+$) as a precursor ion and *m/z* 62 as a product ion in a selected reaction monitoring mode using [$^2\text{H}_8$]anandamide as an internal standard. Anandamide and [$^2\text{H}_8$]anandamide eluted at approximately 6 min, whereas virodhamine eluted at approximately 3.5 min. The lower limit of quantification for both anandamide and virodhamine was 25 pg/ml (8.7 fmol/ml).

Antibody Capture Scintillation Proximity GTP-Binding Assay. [^{35}S]GTPγS binding was measured in a 96-well format, using a modified antibody capture technique previously described (DeLapp et al., 1999). Briefly, Sf9 cell membranes expressing CB1 or CB2 and Gai3β1γ2 (Packard Biosciences, Meriden, CT), drug, and 500 pM [^{35}S]GTPγS (PerkinElmer Life Sciences, Boston, MA) were incubated in a total volume of 200 μl at room temperature for 60 min in GTP-binding assay buffer (20 mM Hepes, 100 mM NaCl, and 5 mM MgCl₂, pH 7.4). The labeled membranes were then solubilized with 0.27% Nonidet P-40 detergent (Roche Applied Science, Indianapolis, IN) for 30 min followed by the addition of a rabbit anti-G_i antibody at a final dilution of 1:400. Following a 60-min incubation at room temperature, anti-rabbit antibody scintillation proximity assay beads (Amersham Biosciences, Piscataway, NJ) were added, and the plates were incubated at room temperature for an additional 3 h. The plates were then centrifuged at 180g for 10 min using a Beckman GS-6R centrifuge (Beckman Coulter, Inc., Fullerton, CA) and counted for 1 min per well using a Wallac 1450 MicroBeta TriLux scintillation counter (PerkinElmer Wallac, Gaithersburg, MD).

Transporter Assay. Inhibition of anandamide transport was measured in RBL-2H3 cells grown in Cytostar T plates (Corning Glassworks, Corning, NY). Briefly, cells were washed with uptake buffer (25 mM HEPES, 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, and 5.6 mM glucose) and then incubated with drug in 0.5% bovine serum albumin for 10 min at room temperature. [^{14}C]Anandamide (5 μM) was then added to the wells, incubated for 2 h, and counted. Nonspecific binding was determined in the presence of 100 μM AM404.

Microdialysis: Animals and Surgery. Adult male Wistar rats (250–300 g; Harlan, Indianapolis, IN) were housed under 12-h light/dark cycle in a temperature-controlled (23°C) animal facility with food and water available *ad libitum*. Rats were anesthetized with a solution containing chloral hydrate and pentobarbital sodium and were mounted in a stereotaxic apparatus (Stoelting Co., Wood Dale, IL) with the incisor bar 3 mm under the horizontal plane passing through the interaural line. Animals were implanted with a guide cannula (Bioanalytical Systems, Inc., West Lafayette, IN) in the lateral striatum

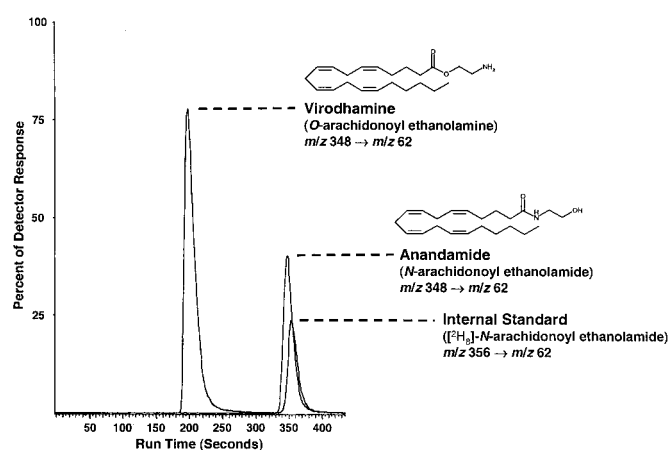


Fig. 1. LC APCI-MS-MS quantification of anandamide and virodhamine. Using *m/z* 348 ($[\text{M} + \text{H}]^+$) as a precursor ion and *m/z* 62 as a product ion in a selected reaction monitoring mode, both anandamide and virodhamine concentrations were quantified. Anandamide and virodhamine were easily separated chromatographically using a C-18 column under isocratic conditions.

(coordinates relative to bregma: anteroposterior = +0.7, mediolateral = +3.5, and dorsoventral = -3.6) according to the atlas of Paxinos and Watson (1986). While the animal was still anesthetized, a commercially available microdialysis probe (Bioanalytical Systems, Inc.) with an active membrane surface of 4.0 mm was inserted through the guide cannula and secured in place. After surgery, rats were housed individually in Plexiglas cages. Principles of laboratory animal care (Guide for the Care and Use of Laboratory Animals, National Academy Press, 1996) were followed, and the Animal Care and Use Committee of Eli Lilly & Co. approved all protocols.

Microdialysis Experiments. The day of the microdialysis experiments, rats were transported from their home cage to the microdialysis testing room. All experiments were performed approximately 24 h postsurgery in awake, freely moving animals during the light period. Microdialysis probes were perfused with a modified Ringer's solution (1.3 mM CaCl₂, 1.0 mM MgCl₂, 3.0 mM KCl, 147.0 mM NaCl, 1.0 mM Na₂HPO₄ × 7H₂O, 0.2 mM NaH₂PO₄ × 2H₂O, and 1% bovine serum albumin, pH 7.2) at a rate of 5.0 μl/min set by a microinfusion pump (SciPro, North Tonawanda, NY). Sample collection started 1 h after the beginning of perfusion. Microdialysis samples were collected every 20 min in polypropylene tubes containing 150 μl of [$^2\text{H}_8$]anandamide standard in methanol. The microdialysis samples were kept at 8°C in a microsampler until the end of the day and

TABLE 1
Tissue levels of virodhamine and anandamide

	Anandamide	Virodhamine	Virodhamine/ Anandamide Ratio
	<i>pmol/g</i>		
Rat			
Hippocampus	22.13 ± 1.38	16.32 ± 0.78	0.73
Cortex	22.55 ± 1.98	20.60 ± 1.63	0.91
Striatum	18.49 ± 6.33	4.24 ± 2.50	0.23
Cerebellum	6.21 ± 2.36	6.91 ± 3.29	1.11
Brainstem	9.41 ± 3.33	4.16 ± 1.95	0.44
Skin	2.12 ± 0.55	6.87 ± 0.26	3.24
Spleen	4.63 ± 0.24	31.60 ± 1.45	6.83
Kidney	10.75 ± 0.13	27.81 ± 0.39	2.58
Ventricle	2.76 ± 0.32	20.11 ± 0.33	7.28
Atria	1.80 ± 0.19	16.80 ± 1.73	9.32
Human			
Hippocampus	16.8 ± 2.9	5.3 ± 0.7	0.32
Rat striatal Microdialysate	None detected (<8.7 fmol/ml)	60 ± 5.7 ^a	Not applicable

^a Values expressed as femtomoles per microliter.

then transferred to a -80°C freezer until further analysis. On completion of the microdialysis experiments, the animals were sacrificed, and their brains were removed and stored in a 10% formalin solution. Each brain was sliced at $50\ \mu\text{m}$ on a cryostat (Leica Microsystems, Inc., Deerfield, IL), stained with Cresyl Violet (Sigma-Aldrich, St. Louis, MO), and examined microscopically to confirm probe placement.

Body Temperature Measurement in Mice. The *in vivo* effects of anandamide and virodhamine were measured in mice by intracerebroventricular injection in 80% DMSO and 20% saline. Rectal temperature was measured 30 min after injection.

Statistics. A Student's *t* test was used to evaluate the *in vivo* effects of anandamide and virodhamine shown in Fig. 5.

Results

The concentration of virodhamine was measured in rat brain and peripheral tissues and human hippocampus (Harvard Brain Tissue Resource Center, Belmont MA; Federal Grant MH/NS 31862) using a LC APCI-MS-MS assay with [$^2\text{H}_8$]anandamide as an internal standard (Figs. 1 and 2). The lower limit of quantitation with this assay was $25\ \text{pg/ml}$ ($8.7\ \text{fmol/ml}$) for both anandamide and virodhamine. Anandamide brain levels measured by this method are comparable with previous reports (Felder et al., 1996). In rat hippocampus, cortex and cerebellum, and human hippocampus, the concentration of virodhamine was similar to anandamide (Table 1). In the brainstem and striatum, the concentration of virodhamine was lower than anandamide. The basal level of virodhamine in microdialysate from rat striatum was $60\ \text{fmol/ml}$; however, anandamide could not be detected ($<8.7\ \text{fmol/ml}$).

In addition to brain, virodhamine was also found in several rat peripheral tissues including skin, spleen, kidney, and heart (Table 1). In all peripheral tissues measured, the concentration of virodhamine was higher than anandamide. In skin, the concentration of virodhamine was more than 3-fold

higher than anandamide. In the spleen, where CB2 receptor expression is high, the concentration of virodhamine was more than 6-fold higher than anandamide. Virodhamine concentration was also higher in rat kidney. In the heart, the concentration of virodhamine was similar in ventricle and atria and more than 7-fold higher than anandamide.

To confirm the role of virodhamine as a cannabinoid receptor ligand, an *in vitro* functional assay was performed. Cannabinoid receptor activity was measured by an antibody-based, G protein-specific [^{35}S]GTP γS binding assay (DeLapp et al., 1999) using Sf9 cell membranes expressing CB1 or CB2 and $\text{G}\alpha\text{i}3\beta\text{1}\gamma\text{2}$ (Packard Biosciences, Meriden, CT) (Fig. 3). EC_{50} values measured in this functional assay matched K_i values reported in the literature for anandamide, 2-AG, and WIN 55,212-2, all of which behaved as full agonists at both receptors (Pertwee, 1999). The Hill slope of all curves at the CB1 receptor was less than unity, which may be a result of the overexpression of receptor and G protein in this Sf9 cell expression system. Interestingly, virodhamine acted as a partial agonist at the CB1 receptor with an EC_{50} of $1906\ \text{nM}$ and an efficacy of 61% compared with anandamide. In contrast, virodhamine was a full agonist at the CB2 receptor with an EC_{50} of $1401\ \text{nM}$ and 100% efficacy compared with anandamide. The potency of virodhamine was similar at both receptors. Virodhamine was less potent than anandamide, 2-AG, and WIN 55,212-2 at both CB1 and CB2.

Virodhamine was also able to inhibit [^{14}C]anandamide transport in RBL-2H3 cells (Fig. 4). The uptake inhibitor, AM404, a derivative of anandamide, was used as a positive control. Other endogenous cannabinoids, including anandamide and the more stable methanandamide, as well as 2-AG, were also tested. The IC_{50} for AM404 was $20\ \mu\text{M}$, which is similar to previous reports (Rakhshan et al., 2000).

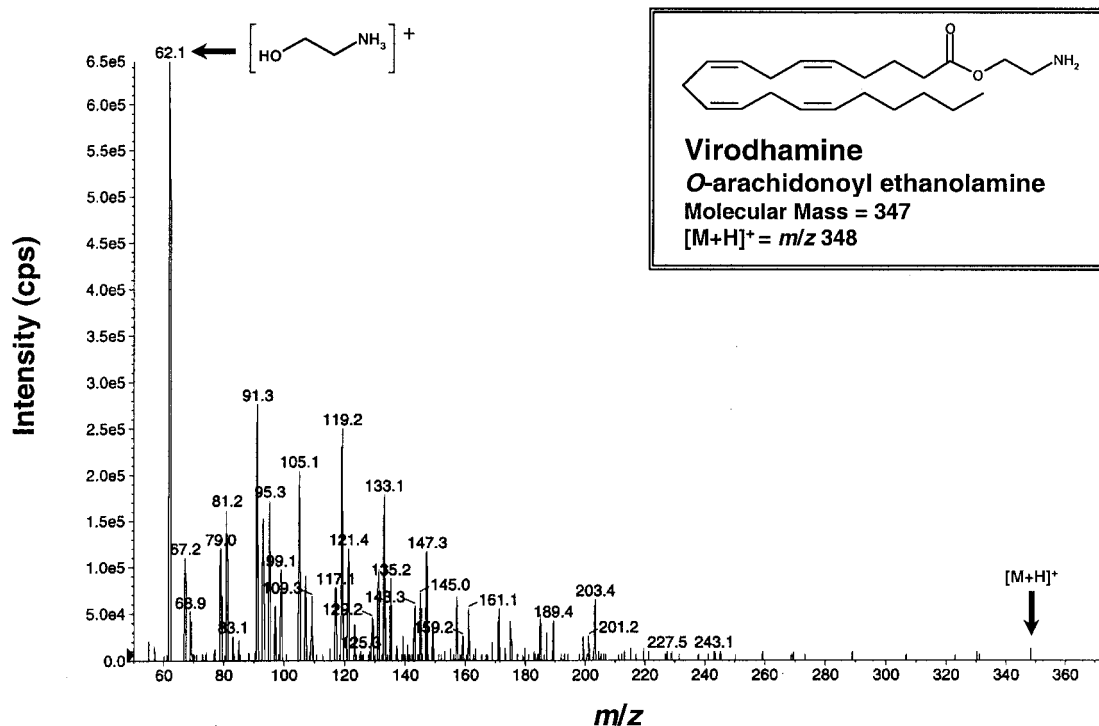


Fig. 2. Daughter ion spectra of virodhamine. Collision-induced dissociation resulted in the formation of an ethanolamine ion ($m/z\ 62$) as the major product ion of virodhamine. This is the same product ion measured for anandamide and the internal standard [$^2\text{H}_8$]anandamide.

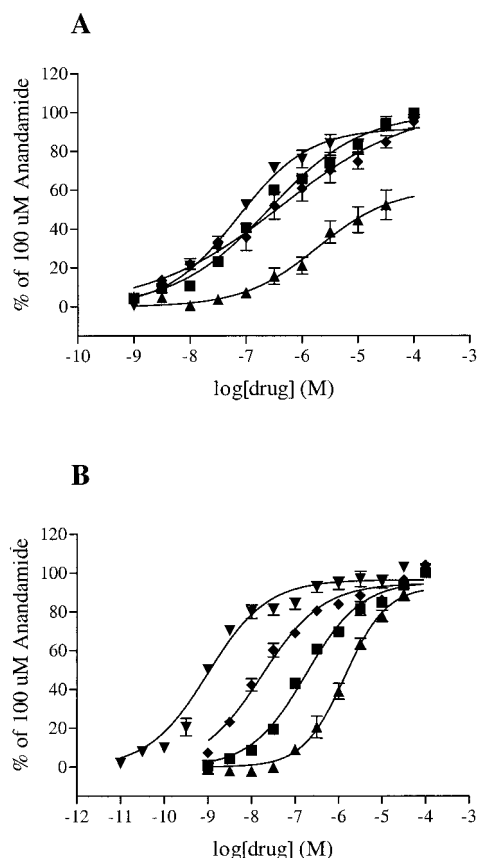


Fig. 3. $G_{\alpha i}$ -specific GTP binding in Sf9 cell membranes expressing the CB1 and CB2 receptor. A, the ability of virodhamine to stimulate CB1 receptor GTP binding was measured in Sf9 cell membranes expressing the receptor and $G_{\alpha i3\beta 1\gamma 2}$. Virodhamine had partial agonist activity at the CB1 receptor with 61% efficacy compared with anandamide. Virodhamine is less potent than the endocannabinoids anandamide and 2-AG and the synthetic agonist WIN 55,212-2. B, the ability of virodhamine to activate CB2 receptors was also tested using GTP binding. Virodhamine was a full agonist at the CB2 receptor with lower potency than anandamide, 2-AG, or WIN 55,212-2. Data are expressed as the average \pm S.E.M. from at least three separate experiments performed in duplicate. ■, anandamide; ▲, virodhamine; ▼, WIN 55,212-2; ◆, 2-AG.

The IC_{50} for anandamide was 34 μ M, and the IC_{50} for 2-AG was 42 μ M. The IC_{50} for virodhamine (123 μ M) was similar to that of methanandamide (130 μ M), and the efficacy of virodhamine was similar to all compounds tested.

The in vivo effects of virodhamine on body temperature in mouse were measured following i.c.v. injection in 80% DMSO and 20% saline and compared with anandamide (Fig. 5). Both compounds were injected i.c.v. because of the likely enzymatic degradation of virodhamine by esterase activity after administration by any other route. Rectal temperature was measured 30 min after injection. Anandamide decreased body temperature by greater than 3°C. Virodhamine decreased body temperature by approximately 2°C. When equal amounts of virodhamine and anandamide were coadministered to mice, the drop in body temperature was not additive. In fact, the decrease was intermediate between anandamide and virodhamine alone.

Discussion

In this report, we describe virodhamine, the first endogenous cannabinoid ligand with antagonist activity at the CB1 receptor. Virodhamine was discovered during development of

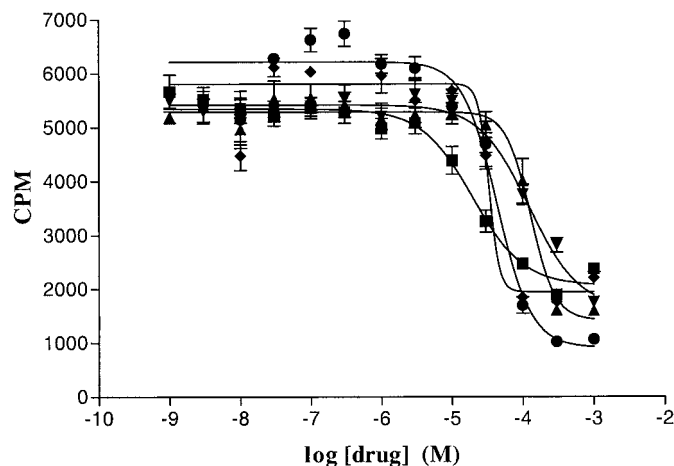


Fig. 4. Inhibition of anandamide transport by virodhamine in RBL-2H3 cells. Virodhamine was tested for its ability to inhibit transport of [14 C]anandamide in RBL-2H3 cells. AM404 inhibited transport with an IC_{50} value of 20 μ M. The endocannabinoids anandamide and 2-AG inhibited transport with IC_{50} values of 34 and 43 μ M. Methanandamide and virodhamine were less potent inhibitors with IC_{50} values of 130 and 123 μ M. Data are expressed as the average \pm S.E.M. from at least three separate experiments performed in duplicate. ■, AM404; ▲, methanandamide; ▼, virodhamine; ●, 2-AG; ◆, anandamide.

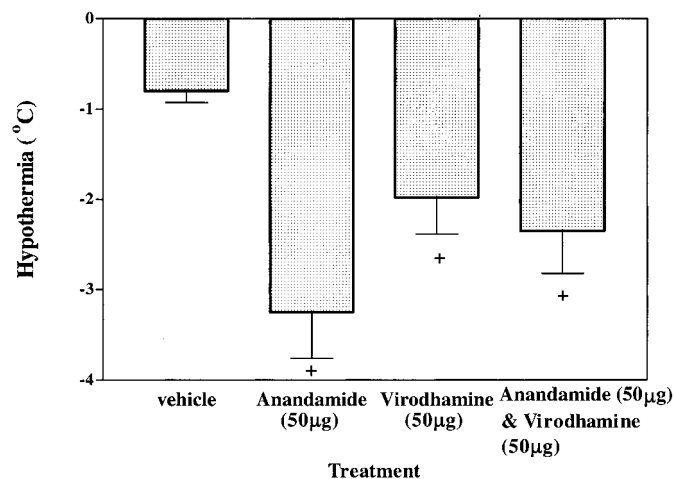


Fig. 5. The effect of virodhamine on body temperature in the mouse. Mice were given virodhamine, anandamide, or vehicle (80% DMSO and 20% saline) by i.c.v. injection, and body temperature was measured 30 min after injection. Anandamide ($n = 10$) lowered body temperature by greater than 3°C. Virodhamine ($n = 10$) had less of a hypothermic effect than anandamide with a decrease of approximately 2°C. When virodhamine and anandamide were coadministered ($n = 4$), the decrease in body temperature was between 2 and 3°C, indicating that virodhamine may be acting as an antagonist in vivo ($p < 0.05$ different from vehicle).

a bioanalytical method to measure anandamide in tissue. A second unexpected analyte peak with the same molecular weight as anandamide but a different retention time was hypothesized to be arachidonic acid and ethanolamine joined by an ester linkage, in contrast to the amide linkage that produces anandamide. The structure of virodhamine was confirmed using an authentic standard that was characterized by both LC APCI-MS-MS and NMR (data not shown). Virodhamine was determined to be an endogenous cannabinoid by its presence in brain and peripheral tissues that express cannabinoid receptors, activity at cannabinoid receptors, blockade of anandamide transport, and antagonism of anandamide-induced hypothermia in mouse.

Tissue measurement of virodhamine and anandamide suggested that levels of these two endogenous cannabinoids are comparable in the brain. In contrast to previous reports (Giuffrida et al., 1999; Walker et al., 1999), we were unable to measure anandamide in rat microdialysate. Because of the greater ionization efficiency of virodhamine compared with anandamide, as well as the relatively short chromatographic separation and similar fragmentation of the two compounds, the possibility exists that previous reports may have been measuring virodhamine, and not anandamide, in rat brain microdialysate.

In the periphery, in contrast to the brain, especially in tissues that express the CB2 receptor, the concentration of virodhamine was higher than anandamide. This suggested the possibility that the efficacy or potency of virodhamine might differ at the CB1 and CB2 receptors. Therefore, a functional measure of GTP binding was used to assess receptor activation by virodhamine. At the CB2 receptor, virodhamine acted as a full agonist. However, virodhamine was a partial agonist at the CB1 receptor with a maximal efficacy of 61% compared with anandamide. Because of its partial agonist activity, virodhamine was tested for antagonist activity at the CB1 receptor in the presence of 1 μ M anandamide in the GTP-binding assay. At high concentrations, virodhamine was able to antagonize the effects of anandamide with a K_b value of 930 nM (data not shown). These data suggest that there are differences in the activation of the two known cannabinoid receptors by virodhamine and that virodhamine may antagonize the effects of other endocannabinoids *in vivo*.

Virodhamine also inhibited anandamide transport, suggesting that the reuptake mechanism could be the same for both of these endocannabinoids. Anandamide transport has been shown to be a saturable, temperature- and time-dependent process that indicates the involvement of a specific transport protein (Rakhshan et al., 2000). In addition, fatty acid amidohydrolase, the enzyme that degrades and inactivates anandamide, has been shown to be required for anandamide transport (Day et al., 2001). Interestingly, in addition to its amidase activity, fatty acid amidohydrolase also has esterase activity (Patricelli and Cravatt, 1999) and could, therefore, also be responsible for virodhamine breakdown *in vivo*.

It is not yet clear how virodhamine is stored, produced, or degraded. However, its ability to block anandamide transport suggests that it may be degraded in a similar manner to anandamide. Virodhamine could be generated from a fatty acid ethanolamine with arachadonic acid in the sn-2 position by a transphosphatidyl transfer reaction catalyzed by phospholipase D. The possibility also exists that virodhamine is produced from anandamide by spontaneous or enzymatically catalyzed chemical rearrangement of the ethanolamine portion of the molecule from an amide linkage to an ester linkage (Markey et al., 2000). Elucidation of these mechanisms will require further experimentation.

A decrease in body temperature is one of the hallmarks of cannabinoid activity *in vivo* (Smith et al., 1994). Virodhamine lowered body temperature in mice, after *i.c.v.* injection, to a level intermediate to that of anandamide. This supports the *in vitro* data, which showed that virodhamine had partial agonist activity at the CB1 receptor. When both endocannabinoids were given together, the decrease in body temperature was less than with anandamide alone. Although the difference in body temperature decrease between anandamide alone and anandamide

and virodhamine together was not statistically significant, the two were clearly not additive. This suggests, as did the *in vitro* data, that virodhamine can act as an endogenous antagonist at the CB1 receptor in the presence of anandamide.

The data presented support a role for virodhamine as an endogenous cannabinoid. This is the first report of an endocannabinoid with partial agonist/antagonist activity at the CB1 receptor and agonist activity at the CB2 receptor. In the periphery, the concentration of virodhamine is higher than anandamide, especially in tissues that express the CB2 receptor. Therefore, the potential exists that virodhamine plays a significant role in the periphery as a full agonist at the CB2 receptor. In the brain, the role of the endocannabinoid system is to modulate neurotransmitter release. This would require an exquisite degree of endocannabinoid control to affect necessary changes in other neuronal signaling systems. The existence of an endogenous antagonist at the CB1 receptor adds a new form of regulation to cannabinoid synapses in the central nervous system.

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References

- Day TA, Rakhshan F, Deutsch DG, and Barker EL (2001) Role of fatty acid amide hydrolase in the transport of the endogenous cannabinoid anandamide. *Mol Pharmacol* **59**:1369–1375.
- DeLapp NW, McKinzie JH, Sawyer BD, Vandergriff A, Falcone J, McClure D, and Felder CC (1999) Determination of [³⁵S]guanosine-5'-O-(3-thio)triphosphate binding mediated by cholinergic muscarinic receptors in membranes from Chinese hamster ovary cells and rat striatum using an anti-G protein scintillation proximity assay. *J Pharmacol Exp Ther* **289**:946–955.
- Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, and Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science (Wash DC)* **258**:1946–1949.
- Felder CC, Briley EM, Axelrod J, Simpson JT, Mackie K, and Devane WA (1993) Anandamide, an endogenous cannabinimimetic eicosanoid, binds to the cloned human cannabinoid receptor and stimulates receptor-mediated signal transduction. *Proc Natl Acad Sci USA* **90**:7656–7660.
- Felder CC, Nielsen A, Briley EM, Palkovits M, Priller J, Axelrod J, Nguyen DN, Richardson JM, Riggan RM, Koppel GA, Paul SM, and Becker GW (1996) Isolation and measurement of the endogenous cannabinoid receptor agonist, anandamide, in brain and peripheral tissues of human and rat. *FEBS Lett* **393**:231–235.
- Giuffrida A, Parsons LH, Kerr TM, Rodriguez de Fonseca F, Navarro M, and Piomelli D (1999) Dopamine activation of endogenous cannabinoid signaling in dorsal striatum. *Nat Neurosci* **2**:358–363.
- Hanus L, Abu-Lafi S, Fride E, Breuer A, Vogel Z, Shalev DE, Kustanovich I, and Mechoulam R (2001) 2-Arachidonyl glyceryl ether, an endogenous agonist of the cannabinoid CB1 receptor. *Proc Natl Acad Sci USA* **98**:3662–3665.
- Markey SP, Dudding T, and Wang TC (2000) Base- and acid-catalyzed interconversions of O-acyl- and N-acyl-ethanolamines: a cautionary note for lipid analyses. *J Lipid Res* **41**:657–662.
- Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S, Martin BR, Compton DR, et al. (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol* **50**:83–90.
- Patricelli MP and Cravatt BF (1999) Fatty acid amide hydrolase competitively degrades bioactive amides and esters through a nonconventional catalytic mechanism. *Biochemistry* **38**:14125–14130.
- Paxinos G and Watson C (1986) *The Rat Brain in Stereotaxic Coordinates*, Academic Press, San Diego, CA.
- Pertwee RG (1999) Pharmacology of cannabinoid receptor ligands. *Curr Med Chem* **6**:635–664.
- Rakhshan F, Day TA, Blakely RD, and Barker EL (2000) Carrier-mediated uptake of the endogenous cannabinoid anandamide in RBL-2H3 cells. *J Pharmacol Exp Ther* **292**:960–967.
- Smith PB, Compton DR, Welch SP, Razdan RK, Mechoulam R, and Martin BR (1994) The pharmacological activity of anandamide, a putative endogenous cannabinoid, in mice. *J Pharmacol Exp Ther* **270**:219–227.
- Walker JM, Huang SM, Strangman NM, Tsou K, and Sanudo-Pena MC (1999) Pain modulation by release of the endogenous cannabinoid anandamide. *Proc Natl Acad Sci USA* **96**:12198–12203.

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