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

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Received December 19, 2001; accepted February 18, 2002 This article is available online at http://jpet.aspetjournals.org

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, virdhamine, with antagonist properties at the CB1 cannabinoid receptor. Virodhamine is arachidonic acid and ethanolamine joined by an ester linkage. Concentrations of virdhamine measured by liquid chromatography-mass spectrometry were 2-9-fold higher than anandamide. In peripheral tissues that express the CB2 cannabinoid receptor, virdhamine concentrations were 2-9-fold higher than anandamide. In contrast to previously described endocannabinoids, virdhamine was a partial agonist with in vivo antagonist activity at the CB1 receptor. However, at the CB2 receptor, virdhamine acted as a full agonist. Transport of [3H]anandamide by RBL-2H3 cells was inhibited by virdhamine. 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, virdhamine 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 Δ9-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 agonists 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 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 virdhamine from the Sanskrit word virdha, which means opposition.

Synthetic virdhamine 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 virdhamine at CB1 and CB2 receptors was measured by antibody capture GTP binding. The ability of virdhamine to decrease body temperature in mouse, a property of CB1 cannabinoid receptor...
ligands, was characterized in vivo. Together, these data indicate that viodhamine acts as a unique endogenous cannabinoid ligand with antagonist activity at the CB1 receptor.

Materials and Methods

Drugs. WIN 55,212-2 \([R\{+\}]-2\text{-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone\) was purchased from Tocris Cookson (St. Louis, MO). AM404 \([N\{4\text{-hydroxyphenyl}\}arachidonylamide\), 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 \((O\text{-arachidonoyl ethanolamine})\) and \(\text{[H}]\text{anandamide}\) 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 viodhamine. Follow ing liquid extraction with organic solvent and further purification by solid phase extraction, samples were separated using a Zorbax Eclipse XDB C-18 column \(75 \times 4.6\text{ mm}\), 5 \(\mu\text{m}\), 3.5 \(\mu\text{m}\), 80 A; 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 viodhamine was accomplished using \(m/z\) 348 \((M + H)^{+}\) as a precursor ion and \(m/z\) 62 as a product ion in a selected reaction monitoring mode using \(\text{[H}]\text{anandamide}\) as an internal standard. Anandamide and \(\text{[H}]\text{anandamide}\) eluted at approximately 6 min, whereas viodhamine eluted at approximately 3.5 min. The lower limit of quantification for both anandamide and viodhamine was 25 pg/ml (8.7 fmol/ml).

Antibody Capture Scintillation Proximity GTP-Binding Assay. \(\text{[35S]}\text{GTP}\gamma\text{S}\) binding was measured in a 96-well format, using a modified antibody capture technique previously described (DeLapp et al., 1999). Briefly, 96 well membranes expressing CB1 or CB2 and Goat/3B1y2 (Packard Biosciences, Meriden, CT), drug, and 500 pM \(\text{[35S]}\text{GTP}\gamma\text{S}\) (PerkinElmer Life Sciences, Boston, MA) were incubated in a total volume of 200 \(\mu\text{l}\) at room temperature for 60 min in GTP-binding assay buffer (20 mM HEPES, 100 mM NaCl, and 5 mM MgCl\(_2\), pH 7.4). The labeled membranes were then solubilized with 0.2% Nonidet P-40 detergent (Roche Applied Science, Indianapolis, IN) for 30 min followed by the addition of a rabbit anti-Gi 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 KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), and 5.6 mM glucose) and then incubated with drug in 0.5% bovine serum albumin for 10 min at room temperature. \(\text{[H]}\text{Anandamide}\) (5 \(\mu\text{M}\)) was then added to the wells, incubated for 2 h, and counted. Nonspecific binding was determined in the presence of 100 \(\mu\text{M}\) AM404.

Microdialysis: Animals and Surgery. Adult male Wistar rats \((250–300 \text{ g}; \text{Harlan, Indianapolis, IN})\) were housed under 12-h light/dark cycle in a temperature-controlled \((23^\circ\text{C})\) 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 (coordinates relative to bregma: anterioposterior = +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 \((1.3 \text{ mM CaCl}_2, 1.0 \text{ mM MgCl}_2, 3.0 \text{ mM KCl}, 147.0 \text{ mM NaCl}, 1.0 \text{ mM Na}_2\text{PO}_4 \times 7\text{H}_2\text{O}, 0.2 \text{ mM NaH}_2\text{PO}_4 \times 2\text{H}_2\text{O}, \text{and 1% bovine serum albumin, pH 7.2})\) at a rate of 5.0 \(\mu\text{l/min}\) set by a microinfusion pump (Scipo, 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 \(\mu\text{l}\) of \(\text{[H]}\text{anandamide}\) standard in methanol. The microdialysis samples were kept at 8°C in a microsampler until the end of the day and analyzed using LC APCI-MS-MS quantification of anandamide and viodhamine. Using \(m/z\) 348 \((M + H)^{+}\) as a precursor ion and \(m/z\) 62 as a product ion in a selected reaction monitoring mode, both anandamide and viodhamine concentrations were quantified. Anandamide and viodhamine were easily separated chromatographically using a C-18 column under isocratic conditions.

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

**TABLE 1**

<table>
<thead>
<tr>
<th>Tissue levels of viodhamine and anandamide</th>
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<tr>
<td>Anandamide</td>
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<td>Rat Hippocampus</td>
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<td>Cortex</td>
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<td>Striatum</td>
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<td>Cerebellum</td>
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| Human Hippocampus | 16.8 ± 2.9 | 5.3 ± 0.7 | 0.32 |

| Rat striatal Microdialysate | None detected | 60 ± 5.7 a | Not applicable |

| Rat striatal Microdialysate | (<8.7 fmol/ml) | | |

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 μm 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 intracebroventricular 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 [2H8]anandamide as an internal standard (Figs. 1 and 2). The lower limit of quantitation with this assay was 25 pg/ml (8.7 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 fmol/ml; however, anandamide could not be detected (<8.7 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 higher 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 [35S]GTPγS binding assay (DeLapp et al., 1999) using Sf9 cell membranes expressing CB1 or CB2 and Gaςβ1γ2 (Packard Biosciences, Meriden, CT) (Fig. 3). EC50 values measured in this functional assay matched Ki 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 EC50 of 1906 nM and an efficacy of 61% compared with anandamide. In contrast, virodhamine was a full agonist at the CB2 receptor with an EC50 of 1401 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 [14C]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 IC50 for AM404 was 20 μM, which is similar to previous reports (Rakhshan et al., 2000).

![Fig. 2. Daughter ion spectra of virdhamine.](image-url)
The IC_{50} for anandamide was 34 μM, and the IC_{50} for 2-AG was 42 μM. The IC_{50} for virodhamine (123 μM) was similar to that of methanandamide (130 μM), 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 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 μM anandamide in the GTP-binding assay. At high concentrations, virodhamine was able to antagonize the effects of anandamide with a K_i 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 transphosphatidylolation 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.

Acknowledgments

We thank the Harvard Brain Tissue Resource Center (Federal Grant MH/NS 31862) for hippocampal tissue samples used to measure anandamide and virodhamine levels.

References


