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N-Oleoyldopamine, a Novel Endogenous Capsaicin-like Lipid That Produces Hyperalgesia*

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***N*-Arachidonoyldopamine (NADA) was recently identified as an endogenous ligand for the vanilloid type 1 receptor (VR1). Further analysis of the bovine striatal extract from which NADA was isolated indicated the existence of substances corresponding in molecular mass to *N*-oleoyldopamine (OLDA), *N*-palmitoyldopamine (PALDA), and *N*-stearoyldopamine (STEARDA). Quadrupole time-of-flight mass spectrometric analysis of bovine striatal extracts revealed the existence of OLDA, PALDA, and STEARDA as endogenous compounds in the mammalian brain. PALDA and STEARDA failed to affect calcium influx in VR1-transfected human embryonic kidney (HEK) 293 cells or paw withdrawal latencies from a radiant heat source, and there was no evidence of spontaneous pain behavior. By contrast, OLDA induced calcium influx (EC_{50} = 36 nM), reduced the latency of paw withdrawal from a radiant heat source in a dose-dependent manner (EC_{50} = 0.72 μg), and produced nocifensive behavior. These effects were blocked by co-administration of the VR1 antagonist iodo-resiniferatoxin (10 nM for HEK cells and 1 μg/50 μl for pain behavior). These findings demonstrate the existence of an endogenous compound in the brain that is similar to capsaicin and NADA in its chemical structure and activity on VR1. Unlike NADA, OLDA was only a weak ligand for rat CB1 receptors; but like NADA, it was recognized by the anandamide membrane transporter while being a poor substrate for fatty-acid amide hydrolase. Analysis of the activity of six additional synthetic and potentially endogenous *N*-acyldopamine indicated the requirement of a long unsaturated fatty acid chain for an optimal functional interaction with VR1 receptors.**

We recently identified *N*-arachidonoyldopamine (NADA)[1] as an endogenous compound that possesses nanomolar potency for vanilloid type 1 (VR1) and cannabinoid CB1 receptors (1–3). Following injection into skin, NADA produces VR1-mediated thermal hyperalgesia, with an EC_{50} of 1.5 μg. Previous studies indicated that anandamide and the lipoxygenase product 12(*S*)-hydroperoxyeicosatetraenoic acid also possess affinity for VR1 (4, 5). However, whereas NADA is similar in potency to capsaicin in a variety of assays of VR1 activity, anandamide and 12(*S*)-hydroperoxyeicosatetraenoic acid are at least 20-fold less potent than capsaicin (4).

All of the putative endovanilloids identified to date are products of arachidonic acid, an unsaturated long chain fatty acid with a primary role in inflammation and pain. Research on the structure-activity relationship for VR1 has shown that a vanillylamine moiety and a long unsaturated acyl chain are required for optimal interaction with the receptor (6, 7). Although anandamide and lipoxygenase products contain the long unsaturated acyl chain, they lack the vanillyl group, which probably accounts for their decreased affinity for and efficacy on VR1 compared with NADA. Because dopamine is a naturally occurring aromatic amine structurally similar to vanillylamine, it was hypothesized that certain unsaturated *N*-acyldopamines may exist in mammalian tissues and function as VR1 ligands (see Fig. 1). NADA, the first such compound to be isolated and identified, produces calcium influx in cultured dorsal root ganglion cells and VR1-transfected human embryonic kidney (HEK) cells in addition to VR1-mediated thermal hyperalgesia. Proposed mechanisms of NADA biosynthesis include the condensation of arachidonic acid with tyrosine and the subsequent conversion of *N*-arachidonoyltyrosine to NADA by tyrosine hydroxylase and L-aromatic amino-acid decarboxylase. NADA is found in multiple regions of the mammalian nervous system, with the highest levels in the striatum, cerebellum, and hippocampus and low levels in the dorsal root ganglion (1). Finally, NADA has been previously described to activate cannabinoid CB1 receptors, but not dopamine D1 and D2 receptors (2, 3). Hence, NADA is the first endogenous vanilloid identified with a structure and potency similar to those of capsaicin, and it is the fifth endocannabinoid to be discovered, the other four (in order of discovery) being anandamide, 2-arachidonoylglycerol, noladin ether, and virodhamine (8–11).

Liquid chromatography/tandem mass spectrometry precursor ion scans of the brain extract in which NADA was identified provided preliminary evidence for the existence of at least

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[1] The abbreviations used are: NADA, *N*-arachidonoyldopamine; VR1, vanilloid type 1 receptor; HEK, human embryonic kidney; OLDA, *N*-oleoyldopamine; PALDA, *N*-palmitoyldopamine; STEARDA, *N*-stearoyldopamine; I-RTX, iodo-resiniferatoxin; AEA, *N*-arachidonoylethanolamine; AMT, anandamide membrane transporter.

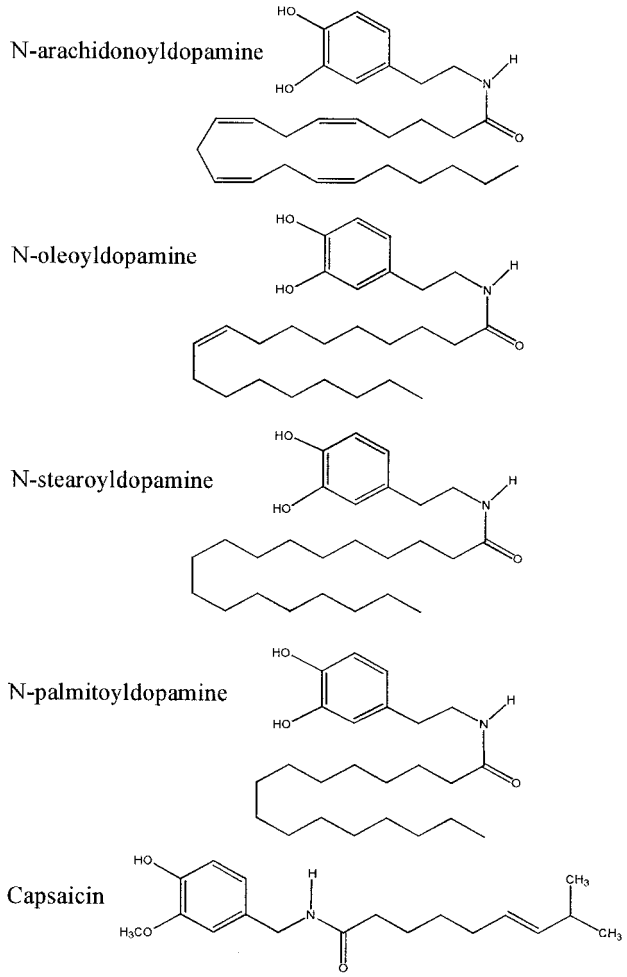

FIG. 1. **Chemical structures of NADA, OLDA, STEARDA, PALDA, and capsaicin.**

three additional *N*-acyldopamines: *N*-oleoyldopamine (OLDA), *N*-palmitoyldopamine (PALDA), and *N*-stearoyldopamine (STEARDA) (Fig. 1). Therefore, we investigated whether these compounds are endogenous to the mammalian brain, their ability to induce VR1-mediated thermal hyperalgesia and to produce calcium influx in VR1-transfected HEK cells, and their binding affinity for cannabinoid CB1 receptors. Here we report that OLDA, PALDA, and STEARDA occur in the mammalian brain and that OLDA is a selective VR1 agonist capable of inducing significant VR1-mediated thermal hyperalgesia with a potency greater than that of NADA while exhibiting affinity for the anandamide transporter, but lacking interaction with fatty-acid amide hydrolase. We also report the efficacy on VR1 of six additional compounds of similar structure.

MATERIALS AND METHODS

Isolation and Identification of Endogenous OLDA, PALDA, and STEARDA—The extraction and purification of OLDA, PALDA, and STEARDA were similar to those described by Huang *et al.* (1) for NADA. Striata were removed from fresh bovine brains and stored at −80 °C until extraction. The tissue was homogenized with a Brinkmann homogenizer (Brinkmann Instruments, Inc., Westbury, NY) in 20 volumes of methanol for 1–1.5 min and centrifuged at 44,000 × *g* for 30 min. Acetone and basified water (20 mM ammonium hydroxide, adjusted to pH 9.1 with acetic acid) were added to the supernatant at a ratio of 1:2:1, and the mixture was centrifuged at 44,000 × *g* for 30 min. The supernatant (80–120 ml) was loaded onto 500-mg phenyl boronic acid Bond-Elut cartridges (Varian, Harbor City, CA) preconditioned with 5 ml of 10% acetic acid in methanol, 5 ml of water, and 2 ml of basified water. Each cartridge was washed with 3 ml of methanol/basified water (75:25), 3 ml of water, 1 ml of acetone, 1 ml of methanol, 1 ml of methanol/basified water (50:50), and 1 ml of water and eluted with 3 ml of 10% acetic acid in methanol. Fractions eluted from multiple cartridges were combined, and ammonium hydroxide (0.125 volumes) was added to the eluant for neutralization, followed by evaporation in a SpeedVac (Savant Instruments, Inc., Halbrook, NY). The extract was desalted following reconstitution first in 0.5 ml of water, which was loaded onto a 100-mg C_{18} Bond-Elut cartridge preconditioned with 0.5 ml of methanol and 0.5 ml of water. The cartridge was washed with 0.5 ml of water, and the remaining extract was reconstituted in 1.6 ml of methanol. 1 ml of water was loaded onto the cartridge reservoir, and the methanol-reconstituted extract was added to the reservoir to form a methanol/water mixture. The cartridge was washed with 1 ml of water and 1 ml of methanol/water (70:30) and eluted with 0.5 ml of methanol. Fractions eluted from multiple samples were combined and evaporated in the SpeedVac, stored at −80 °C, and reconstituted prior to analysis. Structural analysis was accomplished with an MDS Sciex/Applied Biosystems QSTAR Pulsar quadrupole time-of-flight mass spectrometer (qqTOF, Applied Biosystems, Foster City, CA) in negative ion, product ion scanning, and time-of-flight modes. For analysis of chemical behavior of the extract and the synthesized standard, derivatization was performed by addition of equal volumes of anhydrous pyridine and acetic anhydride (Alltech Associates Inc.) to the extract and standard to form *N*-oleoyl-3,4-diacetoxyphenylethylamine. Structural analysis of the derivatized compounds was accomplished using liquid chromatography in tandem with an MDS Sciex/Applied Biosystems API3000 triple quadrupole mass spectrometer in positive ion and multiple reaction monitoring modes.

Synthesis of OLDA, PALDA, and STEARDA and of Their Homologs—OLDA, PALDA, and STEARDA were synthesized from the condensation of the corresponding fatty acyl chlorides with dopamine as previously described (3). The other homologs investigated here were synthesized as follows using the dopamide of ricinoleic acid as an example. To a solution of ricinoleic acid (200 mg, 0.67 mmol) in 1,2-dichloroethane (3 ml) were added triethylamine (383 ml, 270 mg, 2.68 mmol, 4 molar eq), dopamine hydrochloride (127 mg, 0.67 mmol, 1 molar eq), and propylphosphonic acid anhydride (50% EtOAc solution, 511 ml, 0.80 mmol, 1.2 molar eq). After stirring for 2 h at room temperature, the reaction was worked up by evaporation, and the residue was purified by column chromatography (5 g of silica gel) using petroleum ether/EtOAc (6:4) as eluant. 217 mg of the dopamide (75%) was obtained as a colorless oil. The same procedure was employed for retinoic acid (55%), erucic acid (59%), arachidic acid (68%), nonenoic acid (57%), and phenylacetylricinoleic acid (69%). All compounds were fully characterized by spectroscopic techniques (^{1}H and ^{13}C NMR, mass spectrometry, and IR).

Cytosolic Calcium Concentration Assay—Overexpression of human VR1 cDNA in HEK293 cells was carried out as described previously (12). Cells were grown in monolayers in minimal essential medium supplemented with nonessential amino acids, 10% fetal calf serum, and 0.2 mM glutamine and maintained under 95% O_2 and 5% CO_2 at 37 °C. The effect of the substances on the cytosolic Ca^{2+} concentration was determined using Fluo-3, a selective intracellular fluorescent probe for Ca^{2+} (7). 1 day prior to experiments, cells were transferred to six-well dishes coated with poly-L-lysine (Sigma) and grown in the culture medium described above. On the day of the experiment, the cells (50,000–60,000/well) were loaded for 2 h at 25 °C with 4 μM Fluo-3 methyl ester (Molecular Probes, Inc.) in Me_2SO containing 0.04% Pluronic. After loading, cells were washed with Tyrode's solution (pH 7.4), trypsinized, resuspended in Tyrode's solution, and transferred to the cuvette of a PerkinElmer Life Sciences LS50B fluorescence detector under continuous stirring. Due to the rapidity of the calcium response, experiments were carried out by measuring the cell fluorescence at 25 °C (λ_{ex} = 488 nm and λ_{em} = 540 nm) before and immediately after addition of the test compounds at various concentrations. Data are expressed as the concentration exerting a half-maximal effect (EC_{50}). The efficacy of the effect was determined by comparing it with the analogous effect observed with 4 μM ionomycin.

Anandamide Membrane Transport Assay—The effect of compounds on the uptake of *N*-[^{14}C]arachidonoylethanolamine (AEA) by rat basophilic leukemia cells (RBL-2H3) was studied using 3.6 μM [^{14}C]AEA (10,000 cpm) as described previously (13). Cells were incubated with [^{14}C]AEA for 5 min at 37 °C in the presence or absence of varying concentrations (1, 5, 10, and 25 μM) of the inhibitors. Residual [^{14}C]AEA in the incubation medium after extraction with $CHCl_3$/CH_3OH (2:1, by volume), determined by scintillation counting of the lyophilized organic phase, was used as a measure of the AEA that was taken up by cells. Previous studies had shown that, after a 5-min incubation, the amount of [^{14}C]AEA that disappears from the medium of RBL-2H3 cells is found

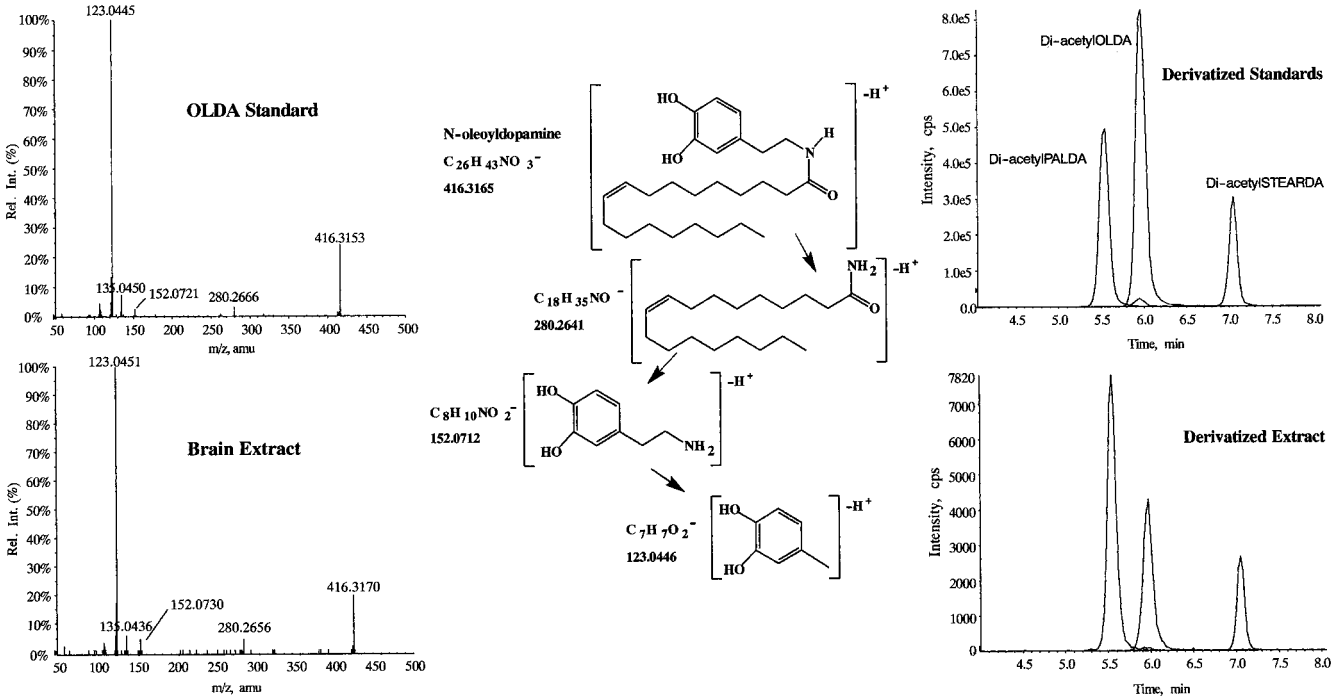

FIG. 2. **Identification of OLDA, PALDA, and STEARDA in bovine striatal extract.** *Left panel*, the synthesized OLDA standard and bovine striatal extract were analyzed in negative ion and product ion scanning modes using a quadrupole time-of-flight mass spectrometer. The spectra show an identical collision-induced fragmentation pattern with the same precursor and fragment ions. Similar results were found for PALDA and STEARDA (data not shown). *Middle panel*, the exact masses of the fragment ions allowed the reconstruction of OLDA. *Right panel*, treatment of the bovine striatal extract and synthetic standards with acetic anhydride and anhydrous pyridine led to the formation of *N*-oleoyl-3,4-diacetoxyphenylethylamine (*Di-acetylOLDA*), *N*-palmitoyl-3,4-diacetoxyphenylethylamine (*Di-acetylPALDA*), and *N*-stearoyl-3,4-diacetoxyphenylethylamine (*Di-acetylSTEARDA*). Multiple reaction monitoring in positive ion mode on a triple quadrupole mass spectrometer revealed coeluting peaks at their respective molecular/fragment masses ($[M + H]^+$ *m/z* 502/196, 476/196, and 504/196). *Rel. Int.*, relative intensity; *amu*, atomic mass units.

mostly (>90%) as unmetabolized [^{14}C]AEA in the cell extract (13). Nonspecific binding of [^{14}C]AEA to cells and plastic dishes was determined in the presence of 100 μM AEA and was never >30%. Data are expressed as the concentration exerting 50% inhibition of AEA uptake (IC_{50}) calculated by GraphPAD.

CB1 Receptor Binding Assay—Displacement assays for CB1 receptors were carried out using [^{3}H]SR141716A (0.4 nM, 55 Ci/mM; Amersham Biosciences) as the high affinity ligand and the filtration technique described previously (2) on membrane preparations (0.4 mg/tube) from frozen male CD rat brains (Charles River Laboratories, Wilmington, MA) in the presence of 100 μM phenylmethylsulfonyl fluoride using varying concentrations (1, 2.5, 5, 10, and 25 μM) of the test compound. Specific binding was calculated with 1 μM SR141716A and was 84.0%.

Fatty-acid Amide Hydrolase Assay—The effect of compounds on the enzymatic hydrolysis of AEA was studied as described previously (13) using membranes prepared from mouse neuroblastoma N18TG2 cells incubated with varying concentrations (1, 5, 10, and 25 μM) of the test compounds and [^{14}C]AEA (9 μM) in 50 mM Tris-HCl (pH 9) for 30 min at 37 °C. [^{14}C]Ethanolamine produced from [^{14}C]AEA hydrolysis was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of $CHCl_3$/CH_3OH (2:1, by volume). Data are expressed as the concentration exerting 50% inhibition of AEA uptake (IC_{50}) calculated by GraphPAD.

Effect of OLDA, PALDA, and STEARDA on Thermal Pain—Male Sprague-Dawley rats weighing 200–250 g served as subjects. They were housed in metal cages in a temperature-regulated (22–23 °C) room with free access to food and water for at least 24 h prior to testing. Artificial lighting was provided from 0600 to 1800 h. On the day of the experiment, each animal was placed in plastic chambers and allowed to acclimate for a 25–30-min period. Following the determination of baseline withdrawal latencies of the hind paw from a radiant heat source, OLDA, PALDA, or STEARDA (0.1, 0.5, 1, 5, 10, and 20 μg) or 50 μl of vehicle (1:1:18 ethanol/emulphor/saline) was injected subcutaneously over a 5–10-s interval into the plantar surface of the left paw of each animal (n = 8–13 per dose). Capsaicin (n = 5–15 per dose: 1, 10, 30, and 50 μg in 50 μl of vehicle) was also tested for comparison purposes. Care was taken to deliver each injection to the center of the paw superficially under the skin so as to minimize tissue injury. Withdrawal latencies were observed at 30, 60, and 90 min after injection. The paw was tested once per time point, and a 20-s cutoff was imposed to prevent tissue damage. The mediation of the effects of these compounds by VR1 receptors was assessed by the intraplantar co-administration of 1 μg of iodo-resiniferatoxin (I-RTX). All dose-response data are expressed as means ± S.E.

Data Analysis—The effect of various doses of OLDA and capsaicin on hyperalgesia across time and the effect of the VR1 antagonist I-RTX on hyperalgesia were analyzed using two-way repeated measures analysis of variance (BMDP Statistical Software, Los Angeles, CA). ED_{50} values from the dose-response data were calculated using the mean change from the base line in the drug- and vehicle-treated animals and analyzed by nonlinear regression with PRIZM (GraphPAD, San Diego, CA). $p < 0.05$ was considered statistically significant.

RESULTS

The identification of OLDA, PALDA, and STEARDA in the bovine striatal extract was performed with an ultrasensitive quadrupole time-of-flight mass spectrometer (qqTOF). Time-of-flight analysis in negative ion mode yielded high precision estimates of the mass of the $M–H^+$ ions for OLDA, PALDA, and STEARDA as 416.3161, 390.3012, and 418.3330, respectively, at the high pressure liquid chromatography retention time specific to each compound. The estimated exact masses permitted the elucidation of the chemical formulas of the compounds in the extract that matched those of OLDA-H ($C_{26}H_{42}NO_3$), PALDA-H ($C_{24}H_{40}NO_3$), and STEARDA-H ($C_{28}H_{44}NO_3$) within 2.2 ppm error. Product ion scans in negative ion mode of the material in the bovine striatal methanol extract and synthetic standards yielded mass spectra with matching precursor and fragment ions (Fig. 2, *left panel*; and Table I). The exact masses of the fragment ions permitted the reconstruction of OLDA (Fig. 2, *center panel*), PALDA, and STEARDA (Table I).

The synthetic standards and bovine striatal extract were treated with acetic anhydride and anhydrous pyridine to examine whether they would undergo the same chemical reaction

TABLE I
Mass measurement of M–H$^+$ and the product ions and structural assignments from bovine striatal extract determined using a quadrupole time-of-flight mass spectrometer

Common fragments	Proposed formulae	Comments
Putative PALDA		
390.3064 *m/z* (5.5 ppm)	$C_{24}H_{40}NO_3$	$M–H^+$
254.2494 *m/z* (1.8 ppm)	$C_{16}H_{32}NO$	$M–H^+ – C_8H_8O_2$
135 *m/z*[a]	$C_7H_7O_2$	$M–H^+ – C_{16}H_{33}NO$
123.0446 *m/z* (4.5 ppm)	$C_7H_7O_2$	$M–H^+ – C_{17}H_{33}NO$
Putative OLDA		
416.3160 *m/z* (2.4 ppm)	$C_{26}H_{42}NO_3$	$M–H^+$
280 *m/z*[a]	$C_{18}H_{34}NO$	$M–H^+ – C_8H_8O_2$
135.0444 *m/z* (5.5 ppm)	$C_8H_7O_2$	$M–H^+ – C_{18}H_{35}NO$
123.0445 *m/z* (5.3 ppm)	$C_7H_7O_2$	$M–H^+ – C_{19}H_{35}NO$
Putative STEARDA		
418.3342 *m/z* (3.6 ppm)	$C_{26}H_{44}NO_3$	$M–H^+$
282 *m/z*[a]	$C_{18}H_{36}NO$	$M–H^+ – C_8H_8O_2$
135.0464 *m/z* (9.2 ppm)	$C_8H_7O_2$	$M–H^+ – C_{18}H_{37}NO$
123.0445 *m/z* (5.3 ppm)	$C_7H_7O_2$	$M–H^+ – C_{19}H_{37}NO$

[a] Due to low signal level, exact mass measurements were not performed.

(acetylation). Multiple reaction monitoring in positive ion mode on a triple quadrupole mass spectrometer revealed coeluting peaks at the expected precursor/fragment masses of $[M + H]^+$ *m/z* 502/196, 476/196, and 504/196, showing that treatment of the synthetic standards and bovine striatal extract led to the formation of *N*-oleoyl-3,4-diacetoxyphenylethylamine, *N*-palmitoyl-3,4-diacetoxyphenylethylamine, and *N*-stearoyl-3,4-diacetoxyphenylethylamine, respectively (Fig. 2, *right panel*). Coeluting peaks were not observed in the untreated extract or reagent controls (data not shown). Therefore, molecules endogenous to the bovine brain with chromatographic behavior, fragmentation pattern, exact masses, and chemical behavior identical to those of OLDA, PALDA, and STEARDA were identified in this study.

The effects of several *N*-acyldopamines and capsaicin on intracellular Ca^{2+} in HEK cells overexpressing human VR1 were examined (Fig. 3 and Table II). Whereas OLDA was as potent as capsaicin and slightly more potent than NADA in this functional assay of VR1 activity, PALDA and STEARDA were nearly inactive. The effect of OLDA was blocked by co-incubation of cells with 10 nM I-RTX, a potent vanilloid receptor antagonist (14). The maximal effect at 10 μM in the presence of the antagonist was 8.1 ± 3.5% of ionomycin (mean ± S.E., $n = 3$). No effect was observed in non-transfected HEK cells (data not shown).

The effect of subcutaneous injections of OLDA, PALDA, STEARDA, or capsaicin into the rat hind paw on withdrawal latencies from a radiant heat source was tested. A significant dose-dependent thermal hyperalgesia was observed after administration of OLDA ($F_{5,62} = 13.02$, $p < 0.0001$, $EC_{50} = 0.72 \pm 0.36$ μg) (Fig. 4), but not PALDA ($F_{4,47} = 2.46$, not significant), STEARDA ($F_{4,47} = 2.06$, not significant), capsaicin ($F_{4,46} = 0.94$, not significant), or vehicle ($F_{1,40} = 0.57$, not significant). The duration of hyperalgesia lasted over 3 h for OLDA. Co-administration of OLDA ($F_{1,13} = 29.12$, $p < 0.0001$) with 1 μg of I-RTX blocked the thermal hyperalgesia (Fig. 5).

Injection of OLDA did not produce immediate nocifensive behavior (licking, lifting, and guarding of the paw) characteristic of capsaicin injection. However, 10–15 min following OLDA administration, animals periodically licked and lifted their paws, which ceased by the time of behavioral testing (30 min after injection). Co-administration of OLDA with 1 μg of I-RTX prevented the occurrence of any nocifensive behavior.

Because NADA was originally found to be a full cannabinoid CB1 receptor agonist (2), OLDA, PALDA, and STEARDA were also tested for their affinity for rat CB1 receptors. None of the three compounds exhibited a strong potency in a binding assay

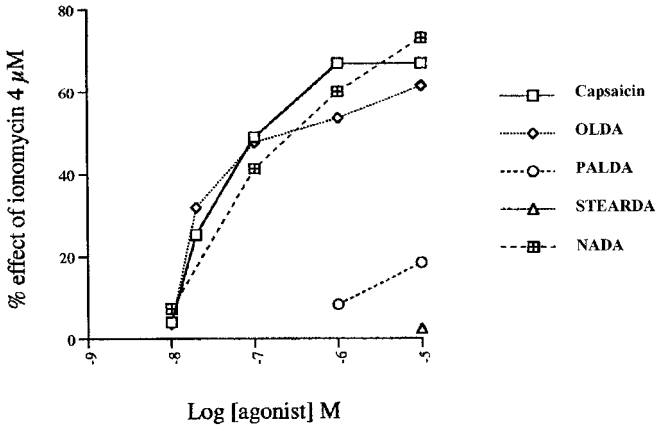

FIG. 3. **Dose-response curves for the effects of capsaicin, OLDA, PALDA, STEARDA, and NADA on cytosolic Ca^{2+} concentration.** Human VR1 was expressed in HEK293 cells, and the effect of capsaicin and *N*-acyldopamines on intracellular Ca^{2+} concentration was determined using Fluo-3, a selective intracellular fluorescent probe for Ca^{2+}. Cell fluorescence before and after addition of test compounds at various concentrations was measured. For comparison of efficacy and potency, the effects of various agonists are expressed as a percent of the effects observed with 4 μM ionomycin. OLDA was as potent as capsaicin and slightly more potent than NADA. PALDA and STEARDA were almost completely inactive. Data are means of three experiments. S.E. values were never >10% of the means and are not shown for the sake of clarity.

carried out with rat brain membranes and [^{3}H]SR141716A as the high affinity CB1 ligand (Table II). Whereas PALDA and STEARDA were inactive at concentrations ≤5 μM, OLDA exhibited some activity, with $K_i = 1.6 \pm 0.4$ μM. Under the same conditions, NADA displayed the highest affinity ($K_i = 0.5 \pm 0.2$ μM), comparable to that previously described for this compound (2, 3).

We investigated the requirements of the fatty acyl chain of *N*-acyldopamines for optimal functional activation of VR1 and tested the functional effect on VR1 of six other potentially endogenous *N*-acyldopamines. We found that, of the six synthetic compounds tested, only those containing at least one double bond exhibited activity in our Ca^{2+} assay (Table II). Saturated *N*-acyldopamines consistently had a low potency, and the potency decreased with increasing length of the acyl chain. The C20:0 derivative (*N*-arachidyldopamine) was inactive, but some efficacy on VR1 at high concentrations could be restored by introducing one double bond, as in *N*-erucyldopamine. Addition of a hydroxy group to OLDA (dopamide of ricinoleic acid) produced a decrease in potency on VR1, which could be restored upon esterification of the hydroxy group with an acetophenyl group (dopamide of 12-phenylacetylricinoleic acid). Notably, the dopamide of retinoic acid, which is a potential naturally occurring metabolite in animals, was the most potent *N*-acyldopamine described so far in our Ca^{2+} assay. This is in agreement with previous analogous observations on "retvanil," the *N*-vanillylamine of retinoic acid (15). Finally, amidation with the same fatty acid chains in capsaicin (dopamide of 8-methyl-Δ^6-*trans*-nonenoic acid) was not sufficient to confer to dopamine a strong potency on VR1, suggesting that, in general, *N*-acyldopamines are less potent than *N*-vanillylamines when the same fatty acid chain is present (compare also olvanil with OLDA and arvanil with NADA) (Table II) (15).

Because OLDA, PALDA, and STEARDA are naturally occurring fatty acid amides, they are potential substrates for fatty-acid amide hydrolase (see Ref. 16 for review) and the anandamide membrane transporter (AMT) (see Ref. 17 for review). Therefore, we tested these three *N*-acyldopamines as possible competitive inhibitors of fatty-acid amide hydrolase and the

TABLE II

Effects of N-acyldopamines and capsaicin on intracellular Ca^{2+} in HEK cells overexpressing human VR1

Data are mean ± S.E. of three independent experiments.

	hVR1[a] (% of effect of 4 μM ionomycin at 10 μM)	hVR1 (EC_{50})	rCB_1 (K_i)
		nM	*μM*
Capsaicin	66.9 ± 3.5	34.0 ± 3.1	>5
NADA (C20:4)	73.1 ± 6.6	63.0 ± 5.5	0.5 ± 0.2
OLDA (C18:1)	62.1 ± 5.5	36.0 ± 0.9	1.6 ± 0.4
STEARDA (C18:0)	6.0 ± 3.1	ND	>5
PALDA (C16:0)	18.4 ± 3.3	ND	>5
ArachidylDA (C20:0)	ND	ND	>5
ErucylDA (C20:1)	58.3 ± 4.8	1585 ± 113	>5
RicinolDA	49.2 ± 3.9	468 ± 41	NT
Phenyl-acetyl-ricinolDA	68.6 ± 5.0	10.0 ± 1.1	NT
CapsDA	57.0 ± 3.6	1000 ± 150	NT
RetinDA	68.1 ± 3.3	6.0 ± 0.8	NT

[a] hVR1, human VR1; rCB1, rat CB1 receptor; ArachidylDA, *N*-arachidyldopamine; ErucylDA, *N*-erucyldopamine; RicinolDA, dopamide of ricinoleic acid; Phenyl-acetyl-ricinolDA, dopamide of 12-phenylacetylricinoleic acid; CapsDA, dopamide of 8-methyl-Δ^6-*trans*-nonenoic acid (the same fatty acyl chain as in capsaicin); RetinDA, dopamide of retinoic acid; ND, not determinable; NT, not tested.

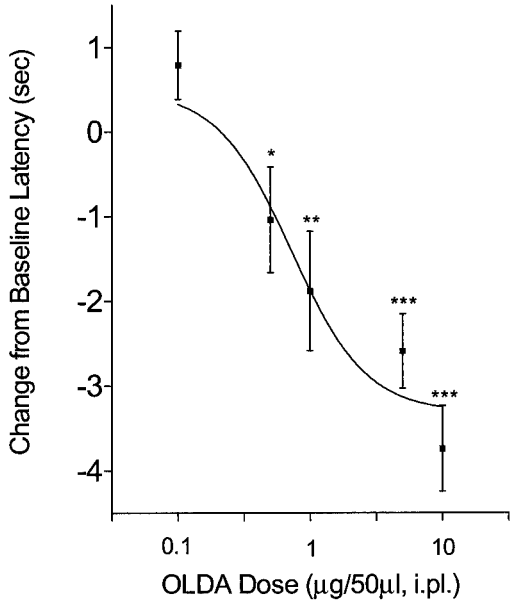

FIG. 4. **OLDA-induced thermal hyperalgesia.** After the determination of baseline withdrawal latencies from a radiant heat source, OLDA was administered subcutaneously over a 5–10-s interval into the rat hind paw (intraplantar), and withdrawal latencies were measured at 30, 60, and 90 min after injection. Injection of OLDA produced a dose-dependent decrease in withdrawal latency. Each point represents the mean change from baseline latency (±S.E., $n = 10$ for each dose) across all time points. The vehicle did not have an effect on withdrawal latency. Nonlinear regression $r^2 = 0.97$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (significant difference from vehicle group; analysis of variance).

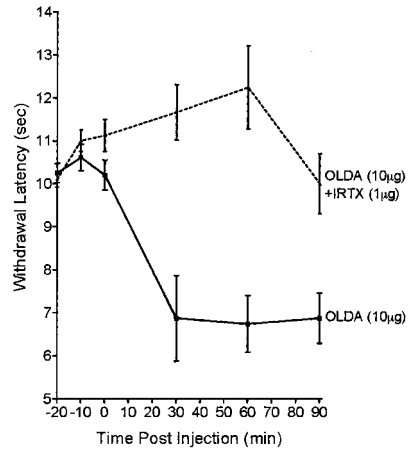

FIG. 5. **Reversal of OLDA-induced thermal hyperalgesia by I-RTX.** I-RTX (1 μg), a highly potent VR1 antagonist, was co-administered with OLDA (10 μg) into the rat hind paw (intraplantar), and withdrawal latency from a radiant heat source was measured. I-RTX completely blocked the thermal hyperalgesia produced by OLDA. Each point represents the mean withdrawal latency (±S.E., $n = 10$ for OLDA and $n = 5$ for OLDA + I-RTX). $p < 0.0001$ (OLDA *versus* OLDA + I-RTX; analysis of variance). The vehicle was inactive (data not shown).

AMT (Table III). None of the compounds significantly inhibited fatty-acid amide hydrolase in mouse N18TG2 cells ($IC_{50} > 25$ μM), whereas OLDA, but not PALDA and STEARDA, appeared to be recognized by the AMT because it inhibited the uptake of [^{14}C]anandamide by RBL-2H3 cells, with $K_i = 14.1 \pm 2.5$ μM (mean ± S.D., $n = 3$). NADA also competed with [^{14}C]anandamide for the AMT ($K_i = 17.3 \pm 7.3$ μM; compare with $K_i = 11.7 \pm 1.0$ μM for anandamide inhibition of [^{14}C]anandamide uptake).

TABLE III

N-Acyldopamines as possible competitive inhibitors of fatty-acid amide hydrolase and AMT

Data are expressed as the concentrations exerting half-maximal inhibition (IC_{50}) and are means ± S.D. three independent experiments. Inhibition of AMT activity was measured by determining the effect on [^{14}C] anandamide uptake by intact RBL-2H3 cells. The effect on fatty-acid amide hydrolase (FAAH) was measured by determining the effect on [^{14}C] anandamide hydrolysis by N18TG2 cell membranes.

	AMT	FAAH
	μM	*μM*
NADA (C20:4)	21.5 ± 9.1[a]	22.0 ± 5.0[a]
OLDA (C18:1)	17.5 ± 3.1	>25
STEARDA (C18:0)	>25	>25
PALDA (C16:0)	>25	>25

[a] Data are from a previous study (9).

DISCUSSION

Mass spectrometric analysis revealed that the *N*-acyldopamines OLDA, PALDA, and STEARDA are produced by mammalian tissues, this conclusion being based upon the identical fragmentation patterns, exact masses, column retention times, and formation of the same diacetyl products upon exposure to derivatizing reagents. OLDA has only a modest affinity for cannabinoid CB1 receptors ($K_i = 1.6$ μM), but it possesses the highest potency of any putative endovanilloid identified to date in the mobilization of intracellular calcium in VR1-transfected HEK cells. Intraplantar injections of OLDA produced dose-dependent thermal hyperalgesia at submicrogram doses, and its effects were blocked by the highly potent VR1 antagonist I-RTX (14). By contrast, as predicted from previous studies showing

lack of VR1 activity in molecules containing a saturated acyl chain (6, 7), PALDA and STEARDA produced neither thermal hyperalgesia after intradermal injections in rats nor calcium mobilization in VR1-transfected HEK cells. The structural requirement of at least one double bond for optimal activation of VR1 is also reflected in the lack of activity of *N*-arachidyldopamine in the Ca^{2+} assay, which was restored, albeit requiring high concentrations, by introducing one double bond to form *N*-erucoyldopamine.

Based upon the EC_{50} values for their effect on Ca^{2+} and the need for ~20 μg of capsaicin intradermally to produce thermal hyperalgesia in rodents (18), the order of potency of the compounds from this series is OLDA > NADA > capsaicin ≫ PALDA = STEARDA (inactive). In the current behavioral experiments, OLDA proved to be at least 30 times more potent than capsaicin. These observations, together with its low affinity for CB1 receptors, suggest that OLDA is both more potent and more selective than NADA as an endogenous agonist of vanilloid *versus* cannabinoid receptors.

The low efficacy of capsaicin was surprising at first glance because Gilchrist *et al.* (18) demonstrated that 10- and 30-μg doses of capsaicin induced a mean decrease in withdrawal latency of ~6 s. This discrepancy could be due to differences in the testing methods because the previous study used 15-min testing intervals with the hind paw receiving four stimuli at each interval, whereas the current study used 30-min testing intervals with the hind paw receiving only one stimulus at each interval. The increased and relatively frequent exposure to noxious heat in the study by Gilchrist *et al.* (18) may have produced central sensitization, which causes a reduced pain threshold and prolonged after-discharges in the spinal targets of the peripheral afferents (19), thus magnifying the effects of capsaicin. Differences in the pharmacodynamics of capsaicin *versus* OLDA may have also influenced the relative measures of potency.

In addition to thermal hyperalgesia, OLDA induced nocifensive behavior that appeared 10–15 min after injection and that consisted of licking and lifting, but not shaking, of the hind paw. This differs from the immediate pungent effect of capsaicin administration, which usually leads to the expression of all three nocifensive behaviors (18). A possible explanation may be differences in channel-gating kinetics, whereby differences in current kinetics have a marked effect on membrane depolarization, action potential generation, and therefore pain perception (20). One example of this phenomenon is provided by olvanil and capsaicin, which are both equally potent in inducing Ca^{2+} uptake by dorsal root ganglion neurons, but only the latter produces nocifensive behavior (20).

The nocifensive behavior induced by OLDA also had a longer duration of action, lasting ~15 min, in contrast to capsaicin, which lasted only 1–3 min in the study by Gilchrist *et al.* (18). Similarly, the duration of the thermal hyperalgesia induced by OLDA lasted at least 3 h, in comparison with the 45-min effect of capsaicin (18). This suggests that OLDA may have a slower rate of metabolic inactivation, providing for a longer period of receptor activation. Previous studies showed that, compared with anandamide, NADA is metabolized at a much slower rate by fatty-acid amide hydrolase, the major enzyme catalyzing long chain fatty acid amide hydrolysis (1, 2). We found that OLDA was even less potent than NADA as an inhibitor of this enzyme, suggesting that it is also a very poor substrate and is likely to have a longer half-life *in vivo*.

Because we found here that OLDA (but not PALDA or STEARDA) is capable of inhibiting anandamide uptake by intact cells, the AMT is a possible inactivating protein capable of recognizing this compound. However, it must be pointed out that, because the binding site on VR1 for capsaicin, anandamide, and other agonists is intracellular (21–23), the AMT may play a facilitative role in the effect of extracellular OLDA (and NADA) on VR1. On the other hand, because the AMT also facilitates the release of its substrates from the cell (24), this protein may also mediate the inactivation of the effect of intracellular OLDA (and NADA) on VR1 (25).

The capsaicin-like activity of NADA and OLDA observed in the radiant heat test and the previous identification of NADA and now of OLDA in bovine nervous tissue suggest that these compounds may function as endogenous activators of VR1 or "endovanilloids" (1). A previous study using rat brain homogenates and incubation with potential precursors revealed that NADA can be synthesized through a condensation reaction between arachidonic acid and dopamine or may be formed from the condensation of arachidonic acid with tyrosine and then converted to NADA by the enzymes tyrosine hydroxylase and L-aromatic amino-acid decarboxylase (1). Furthermore, NADA can be inactivated by conversion into the less active 3-*O*-methyl-NADA by catechol *O*-methyltransferase (1). Because of the similar chemical structures of NADA and OLDA, these are all possible mechanisms whereby OLDA may be produced or inactivated, although more work is needed to establish these mechanisms.

OLDA may function as either a peripheral or central mediator of VR1 activation. Although the subject of VR1 receptors in brain has been controversial, mounting evidence indicates that VR1 receptors are, in fact, produced by the brain and are functional (26, 27) (see Refs. 25 and 28 for reviews). Several putative central nervous system-mediated actions of VR1 receptors have been postulated, including modulation of motor function, body temperature, and pain (29–33). Notably, Palazzo *et al.* (33) found that microinjections of capsaicin into the periaqueductal gray matter caused analgesia to the same type of thermal stimulus used here, an effect opposite to that observed following intradermal injections. They proposed that presynaptic stimulation of glutamate release was responsible for the effect. These observations are consistent with the results of whole cell patch clamp recordings of locus ceruleus neurons reported by Marinelli *et al.* (34). These investigators found VR1-mediated increases in the frequency of miniature excitatory postsynaptic potentials in the locus ceruleus neurons, which, along with other data, suggests that VR1 receptors exert presynaptic facilitation of the release of glutamate.

Although PALDA and STEARDA were inactive in our tests of thermal hyperalgesia and intracellular Ca^{2+}, the presence of these compounds in the brain suggests they have a physiological function. In this respect, it is noteworthy that STEARDA was found to inhibit arachidonate 5-lipoxygenase, with an IC_{50} of 16 nM, suggesting that it may function endogenously to inhibit the formation of leukotrienes (35).

Alternatively, PALDA and STEARDA may act in concert with other *N*-acyldopamines or similar lipids. As noted by Mechoulam *et al.* (36), lipid mediators are often synthesized and released together and can produce "entourage effects," *i.e.* a heightening of the effects of the major bioactive compound. Such entourage effects of saturated *N*-acylethanolamines on anandamide stimulation of human vanilloid receptors have been observed (37–39). *N*-Lauroylethanolamine and *N*-palmitoylethanolamine produced a leftward shift in the anandamide dose-response curve in VR1-mediated Ca^{2+} influx assays by increasing the potency of anandamide by ~4–10-fold at the highest concentrations used (10 μM) (37, 38). *N*-Palmitoylethanolamine produced a 2-fold decrease in the K_i value for anandamide binding to VR1 receptors (37). Finally, *N*-palmitoylethanolamine also enhances the antiproliferative effects on breast

cancer cells of both anandamide and capsaicin, which are mediated by CB1 and VR1 receptors, respectively (39, 40). Therefore, it is plausible that PALDA and STEARDA potentiate the activation of VR1 by NADA and OLDA.

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LIPIDS AND LIPOPROTEINS:

N-Oleoyldopamine, a Novel Endogenous Capsaicin-like Lipid That Produces Hyperalgesia

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