

# An endogenous capsaicin-like substance with high potency at recombinant and native vanilloid VR1 receptors

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Edited by L. L. Iversen, University of Oxford, Oxford, United Kingdom, and approved April 18, 2002 (received for review April 3, 2002)

The vanilloid receptor VR1 is a nonselective cation channel that is most abundant in peripheral sensory fibers but also is found in several brain nuclei. VR1 is gated by protons, heat, and the pungent ingredient of “hot” chili peppers, capsaicin. To date, no endogenous compound with potency at this receptor comparable to that of capsaicin has been identified. Here we examined the hypothesis, based on previous structure-activity relationship studies and the availability of biosynthetic precursors, that *N*-arachidonoyl-dopamine (NADA) is an endogenous “capsaicin-like” substance in mammalian nervous tissues. We found that NADA occurs in nervous tissues, with the highest concentrations being found in the striatum, hippocampus, and cerebellum and the lowest concentrations in the dorsal root ganglion. We also gained evidence for the existence of two possible routes for NADA biosynthesis and mechanisms for its inactivation in rat brain. NADA activates both human and rat VR1 overexpressed in human embryonic kidney (HEK)293 cells, with potency ( $EC_{50} \approx 50$  nM) and efficacy similar to those of capsaicin. Furthermore, NADA potently activates native vanilloid receptors in neurons from rat dorsal root ganglion and hippocampus, thereby inducing the release of substance P and calcitonin gene-related peptide (CGRP) from dorsal spinal cord slices and enhancing hippocampal paired-pulse depression, respectively. Intradermal NADA also induces VR1-mediated thermal hyperalgesia ( $EC_{50} = 1.5 \pm 0.3$   $\mu$ g). Our data demonstrate the existence of a brain substance similar to capsaicin not only with respect to its chemical structure but also to its potency at VR1 receptors.

Vanilloid receptors of type 1 (VR1) are nonselective cation channels, expressed in peripheral sensory C and A $\delta$  fibers and gated by nociceptive stimuli such as low pH, heat, and some plant toxins, of which capsaicin, the pungent principle of chili peppers, is the best known example (1–4). Evidence obtained by several laboratories and using different techniques (5–10) showed that VR1 is present also in the central nervous system, where it is unlikely to be the target of noxious heat and low pH, thus suggesting the existence of brain endogenous agonists for this receptor (11). Indeed, lipid mediators previously known to serve other functions in the brain, i.e., the endocannabinoid anandamide and some lipoxygenase derivatives, activate VR1, albeit with a potency considerably lower than that of capsaicin (12–14). The antinociceptive effects of VR1 blockers in two models of inflammatory hyperalgesia (15, 16) suggest that “endovanilloids” might be produced also by peripheral tissues and act in concert with locally enhanced temperature and acidity during inflammation.

If an endovanilloid did exist, what would be the structural prerequisites that would allow for an optimal interaction with vanilloid receptors? Structure-activity relationship studies for vanilloid receptors have indicated that both the vanillyl-amine moiety and a long, unsaturated acyl chain are necessary to achieve an

optimal functional interaction with the binding site (17, 18). Accordingly, anandamide and lipoxygenase products (12–14) contain the requisite acyl chain but lack the vanillyl group, hence their relatively low potency at VR1. The only two metabolites found in mammals that are similar structurally to vanillyl-amine are dopamine and its 3-*O*-methyl derivative, homovanillyl-amine. Thus, unsaturated *N*-acyl-dopamines and *N*-acyl-homovanillyl-amines might behave as VR1 agonists. The existence in mammals of *N*-acyl-dopamines was hypothesized in earlier studies (19) but never investigated. Among them, *N*-arachidonoyl-dopamine (NADA) was synthesized and shown to bind to cannabinoid but not dopamine receptors (20), making it a good candidate endovanilloid by analogy with anandamide. Therefore, in the present study we synthesized NADA and *N*-arachidonoyl-homovanillyl-amine (3-*O*-methyl-NADA; Fig. 1) and tested their activity in human embryonic kidney (HEK)293 cells transfected with human or rat VR1 cDNAs. More importantly, by exploiting high-sensitivity and -accuracy mass spectrometric (MS) techniques, we looked for NADA in both rat and bovine nervous tissue. We report that NADA is a naturally occurring substance similar to capsaicin not only with regard to its chemical structure but also to its potency at VR1 receptors.

## Methods

**Chemical Synthesis.** NADA and *O*-methyl-NADA were synthesized from the corresponding amines and arachidonoylchloride (Sigma) as described (18, 20). The compounds were purified by column chromatography, and their chemical structure was confirmed by proton NMR and infrared spectroscopy. [<sup>3</sup>H]NADA [1 mCi/mol (1 Ci = 37 GBq)] was synthesized from arachidonoylchloride and [<sup>3</sup>H]dopamine (NEN, 53.6 Ci/mmol) by using the same procedure.

**Intracellular Ca<sup>2+</sup> Concentration Assays with HEK293 Cells Transfected with VR1.** HEK293 cells transfected with either rat VR1 or human VR1 cDNA (10, 13) were kindly provided by John Davis, Glaxo SmithKline, and grown as monolayers in minimum essential medium supplemented with nonessential amino acids/10% FCS/0.2 mM glutamine and maintained under 95%/5% air/CO<sub>2</sub> at 37°C. The effect of the substances on [Ca<sup>2+</sup>]<sub>i</sub> was determined by using Fluo-3, a selective intracellular fluorescent probe for Ca<sup>2+</sup>. Cells (50–60,000 per well) were loaded for 2 h at 25°C with 4  $\mu$ M Fluo-3

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: VR1, vanilloid receptor of type 1; NADA, *N*-arachidonoyl-dopamine; HEK, human embryonic kidney; DRG, dorsal root ganglion/ganglia; CGRP-LI, calcitonin gene-related peptide-like immunoreactivity; SP-LI, substance P-like immunoreactivity; PS, population spike.

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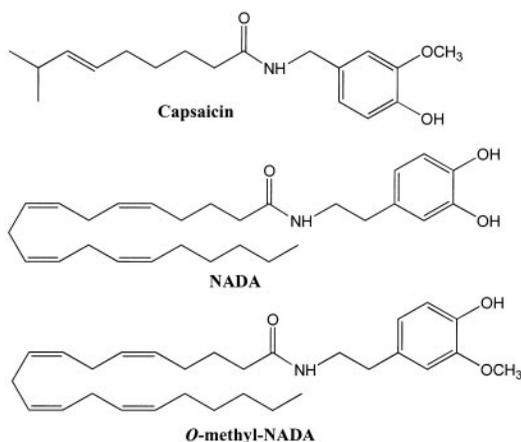


Fig. 1. Chemical structures of capsaicin, NADA, and O-methyl-NADA.

methylester (Molecular Probes) and transferred to the cuvette of the fluorescence detector (Perkin–Elmer LS50B) under continuous stirring. Fluorescence was measured at 25°C ( $\lambda_{EX} = 488$  nm,  $\lambda_{EM} = 540$  nm) before and after the addition of the test compounds at various concentrations. In some experiments cells were pretreated for 5 min with phorbol 12,13-dibutyrate (100 nM, Sigma) in the presence or absence of bis-indolmaleimide (0.2  $\mu$ M, Sigma).

#### Isolation, Identification, and Quantification of Endogenous NADA.

Striata were removed from fresh bovine brains and stored at  $-80^{\circ}\text{C}$  until extraction. The tissue was homogenized in 20 vol of ice-cold methanol for 45 s to 1 min and centrifuged at  $44,000 \times g$  for 30 min. Acetone and basified water (20 mM ammonium hydroxide pH-adjusted to 9.14 with acetic acid) were added to the supernatant in the ratio of 1:2:1, and the mixture was centrifuged at  $44,000 \times g$  for 30 min. The supernatant was loaded onto preconditioned (10% acetic acid in methanol, water, and basified water) PBA Bond-Elut cartridges (Varian), and washed with methanol/basified water (75:25)/water/acetone/methanol/methanol/basified water (50:50)/water and eluted with 10% acetic acid in methanol. Fractions eluted from multiple cartridges were combined and evaporated in a Speed Vac (Savant) and stored at  $-80^{\circ}\text{C}$ . The final extract was reconstituted in 200  $\mu$ l of 10% acetic acid in methanol and centrifuged at  $36,220 \times g$  for 3 min. MS analysis was accomplished with an Applied Biosystems/MDS Sciex API3000 triple quadrupole or a Pulsar quadrupole time of flight, or the extract was derivatized as follows: a portion of the bovine striatal extract was diluted 10-fold, to which equal volumes of anhydrous pyridine and acetic anhydride (Alltech Associates) were added. The levels of NADA in various male rat brain regions were analyzed by using multiple-reaction monitoring mode on the liquid chromatography/tandem MS system. Recovery was calculated from brain homogenate treated with the NADA standard.

**Biosynthesis and Inactivation of NADA.** The formation of [ $^3\text{H}$ ]NADA was observed by incubating rat brain whole homogenates, obtained by homogenization of adult male rat brain (Sprague–Dawley) in 50 mM Tris-HCl, pH 7.4, with 50  $\mu$ M arachidonic acid, 1  $\mu$ Ci/ml [ $^3\text{H}$ ]arachidonic acid, and 50  $\mu$ M tyrosine or dopamine for 30 min at  $37^{\circ}\text{C}$ . Control incubations were carried out with heat-inactivated homogenates. After the incubation, the mixtures were extracted with 2 vol of chloroform/methanol (2:1), and the organic phase was lyophilized and analyzed by thin layer chromatography on analytical silica plates (Merck) first developed with chloroform/methanol/acetic acid and then with the organic phase of a mixture of ethyl acetate/water/2,2,4-tri-methylpentane/acetic acid (55:50:25:10 by

vol, solvent B). NADA mobility in the two cases was  $R_f = 0.45$  and  $R_f = 0.8$ . In the case of incubations with tyrosine, in one set of experiments the catechol-*O*-methyltransferase inhibitor OR-486, at a concentration (30  $\mu$ M) active also against tyrosine  $\beta$ -hydroxylase (21), was included.

The metabolism of [ $^3\text{H}$ ]NADA to [ $^3\text{H}$ ]dopamine or *O*-methyl- $^3\text{H}$ ]NADA was studied by incubating  $10,000 \times g$  pellets (mitochondria),  $100,000 \times g$  pellets (microsomes), or cytosolic fractions from rat brain or liver homogenates in phosphate buffer at pH 7.0 or 9.0 with [ $^3\text{H}$ ]NADA (5  $\mu$ M) for 1 h at  $37^{\circ}\text{C}$ . After the incubation, the mixtures were extracted with 2 vol of chloroform/methanol (2:1). The aqueous phase containing [ $^3\text{H}$ ]dopamine was counted directly, whereas the organic phase, containing unreacted [ $^3\text{H}$ ]NADA and *O*-methyl- $^3\text{H}$ ]NADA, was lyophilized and purified by thin layer chromatography developed with solvent B (the  $R_f$  of *O*-methyl-NADA was 0.95). The formation of *O*-methyl-NADA also was confirmed by analysis of the lipid extract by atmospheric pressure chemical ionization/liquid chromatography MS. In some experiments the catechol-*O*-methyltransferase inhibitor OR-486 (1  $\mu$ M) (21) was included in the incubation mixture and inhibited the formation of *O*-methyl-NADA by  $55 \pm 9\%$  (mean  $\pm$  SD,  $n = 3$ ).

The uptake of [ $^3\text{H}$ ]NADA by intact C6 glioma cells was studied as described (22). Nonspecific uptake was determined by incubating the cells at  $4^{\circ}\text{C}$  or at  $37^{\circ}\text{C}$  in the presence of 50  $\mu$ M anandamide, both of which reduced the uptake of [ $^3\text{H}$ ]NADA by  $\approx 45\%$ .

#### Ca<sup>2+</sup> Fluorescence Measurements in Dorsal Root Ganglion (DRG) Neurons.

DRG were removed from rats (1–3 days old) as reported previously (23). Cells were plated on poly-L-lysine and laminin-coated glass coverslips and loaded with Fura-2 methylester (3  $\mu$ M). Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) fluorescence measurements were performed at  $37^{\circ}\text{C}$  with a Nikon eclipse TE300 microscope. Fura-2 methylester was excited at 340 and 380 nm to indicate relative [Ca<sup>2+</sup>]<sub>i</sub> changes by the  $F_{340}/F_{380}$  ratio recorded with a dynamic image-analysis system (LABORATORY AUTOMATION 2.0, RBR-Altair, Florence, Italy).

#### Effect of NADA on Neuropeptide Release from DRG Slices.

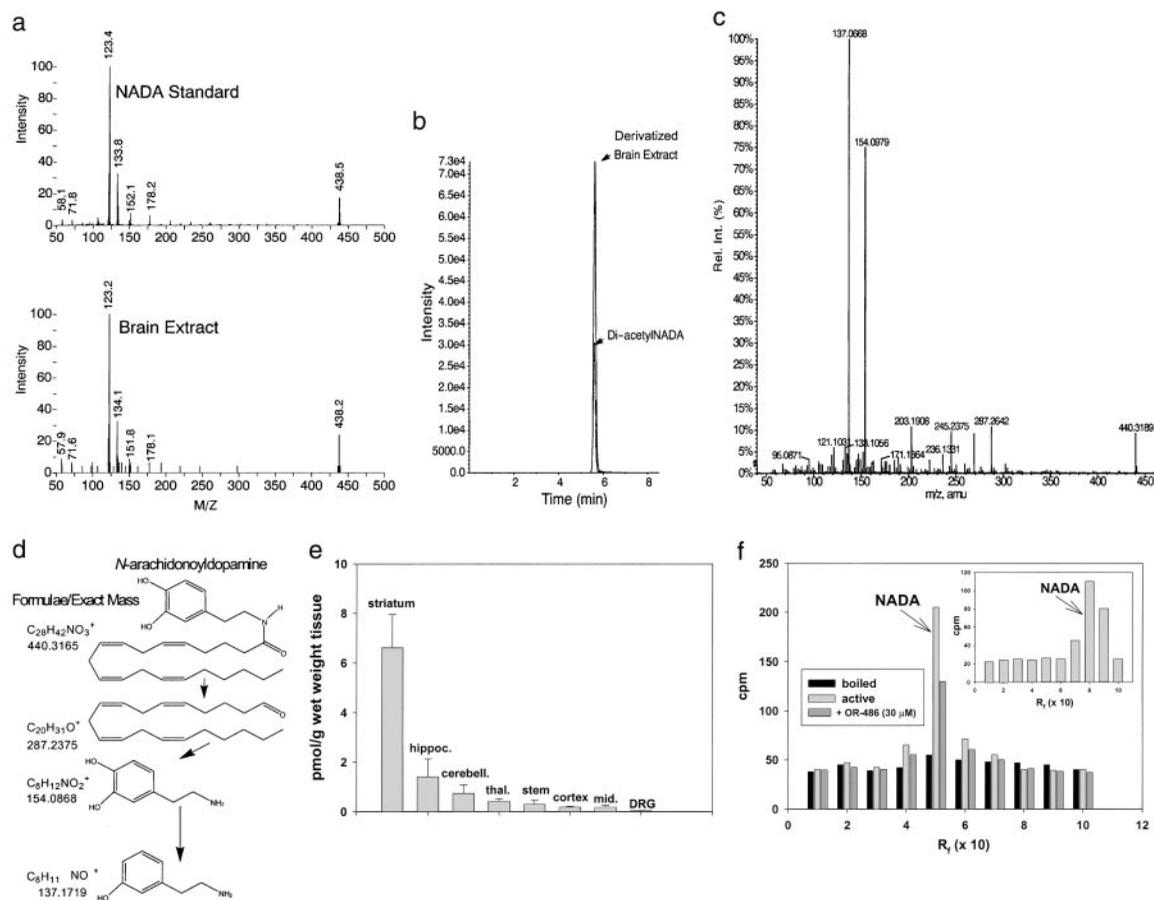
Thick slices ( $\approx 0.4$  mm) from the dorsal part of the cervical and lumbar enlargements of the adult rat spinal cord were prepared as shown previously (23). Slices transferred to 2-ml chambers were superfused with a  $37^{\circ}\text{C}$  and oxygenated Krebs solution with added 0.1% BSA, 1  $\mu$ M phosphoramidon, and 1  $\mu$ M captopril. Freeze-dried fractions were reconstituted with assay buffer and analyzed by enzyme immunoassays for calcitonin gene-related peptide and substance P-like immunoreactivity (CGRP-LI and SP-LI, respectively) as described (23).

#### Effect of NADA on Thermal Hyperalgesia.

Subsequent to determination of baseline withdrawal latencies of the hind paw from a radiant heat source, NADA (0.1, 0.5, 1, 5, or 10  $\mu$ g or vehicle, 1:1:18 ethanol/emulphor/saline, 50  $\mu$ l) was injected in the plantar (i.p.) surface of the paws of male Sprague–Dawley rats. Withdrawal latencies were observed at 10, 30, 50, and 90 min after injection. The mediation of the hyperalgesic effect of NADA by VR1 receptors was assessed by i.p. injection of 0.7 mg of capsazepine, 1  $\mu$ g of iodo-resiniferatoxin, or vehicle 10 min before injection of 5  $\mu$ g of NADA. ED<sub>50</sub> values from the dose-response data were estimated by using the mean change from baseline in the drug- and vehicle-treated animals by nonlinear regression with PRIZM (GraphPad, San Diego). The effect of capsazepine and iodo-resiniferatoxin on NADA hyperalgesia was assessed by repeated measures ANOVA (BMDP Statistical Software).

#### Effect of NADA on Hippocampal Paired-Pulse Depression.

Transverse hippocampal slices (400  $\mu$ m) were prepared from young-adult Sprague–Dawley rats (4–6 weeks old) and maintained in an interface-type recording chamber perfused with artificial cere-



**Fig. 2.** Identification and biosynthesis of NADA in brain. (a) Identical mass spectra of NADA and material in bovine striatal extract. Standard and extract were analyzed in negative-ion, product-ion scanning mode by using a triple-quadrupole mass spectrometer. (b) Purified extract of bovine striatum and synthetic NADA were treated with acetic anhydride to form *N*-arachidonoyl-3,4-diacetoxyphenylethylamine (di-acetylNADA). Multiple-reaction monitoring on a triple-quadrupole mass spectrometer revealed coeluting peaks with molecular/fragment ions 524/196 atomic mass units. (c) Quadrupole time-of-flight MS analysis of material in the brain extract in positive-ion mode yielded a mass estimate of 440.3189, which is within 4.6 ppm of the mass of NADA. Exact masses of fragment ions permitted reconstruction of NADA as shown in *d*. (e) Distribution of NADA (pmol/g wet weight tissue, means  $\pm$  SEM,  $n = 4$ –12) in central and peripheral nervous system. hippoc., hippocampus; cerebell., cerebellum; thal., thalamus, mid., midbrain. (f) Biosynthesis of [ $^3$ H]NADA from [ $^3$ H]arachidonic acid (50  $\mu$ M) and tyrosine (50  $\mu$ M) in rat brain homogenates. Lipids from different incubates (boiled homogenate, enzymatically active homogenate, and homogenate in the presence of the catechol-*O*-methyltransferase inhibitor OR-486 at 30  $\mu$ M (a concentration that blocks the conversion of tyrosine into dopamine)) were extracted and purified by thin layer chromatography. A peak sensitive to OR-486 with the same  $R_f$  as synthetic NADA was scraped from the plate and reanalyzed under different eluting conditions (*Inset*). Similar results (except for the effect of OR-486) were obtained when incubating [ $^3$ H]arachidonic acid (50  $\mu$ M) and dopamine (50  $\mu$ M). The data are representative of three experiments.

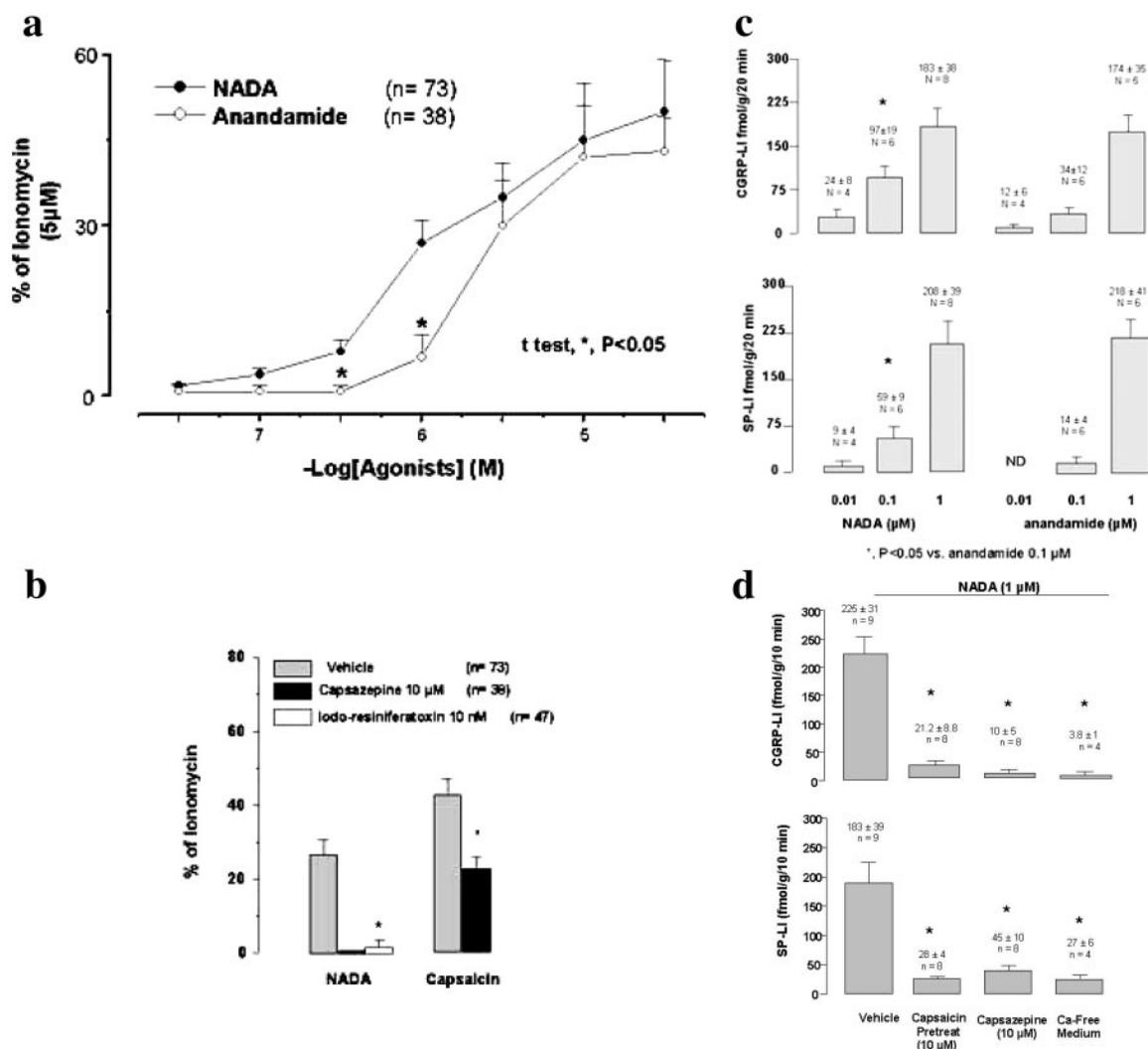
brospinal fluid at 1.5 ml/min and 29–30°C. A glass extracellular recording electrode was placed in *stratum pyramidale* of the CA1 region to record population spikes (PSs) evoked by a bipolar stimulating electrode placed in *stratum radiatum* at the CA1–CA3 border. The stimulus strength was adjusted to evoke a PS of approximately half-maximal amplitude, and paired-pulse stimulation at intervals between 5 and 200 ms was used to identify effects on both the amplitude of the first (PS1) and second (PS2) PSs (24). Drugs were applied by addition to the perfusion medium, and statistical tests were made with Student's paired *t* test, in which  $P < 0.05$  was considered significant.

## Results and Discussion

In HEK293 cells overexpressing human or rat VR1, NADA induced a strong enhancement of intracellular  $Ca^{2+}$  ( $EC_{50} = 40 \pm 6$  and  $48 \pm 7$  nM, maximal stimulation at 10  $\mu$ M =  $73.1 \pm 6.4$  and  $69.6 \pm 5.2\%$  of the effect of 4  $\mu$ M ionomycin, respectively, means  $\pm$  SD,  $n = 4$ ) that was abolished by the VR1 antagonist, iodoresiniferatoxin (10 nM of which reduced the maximal effect on human VR1 to  $9.5 \pm 4.1\%$  of the effect of ionomycin) and was negligible in nontransfected cells ( $5.5 \pm 2.9\%$  of the effect of

ionomycin at 10  $\mu$ M). At either human or rat VR1, capsaicin was slightly more potent than NADA ( $EC_{50} = 26 \pm 8$  and  $33 \pm 7$  nM, respectively,  $n = 6$ ), whereas anandamide was  $\approx 10$ -fold less potent ( $EC_{50} = 550 \pm 85$  and  $465 \pm 33$  nM, respectively,  $n = 6$ ). The 3-*O*-methyl-NADA ( $EC_{50} = 126 \pm 19$  and  $309 \pm 25$  nM, at human or rat VR1, respectively,  $n = 3$ ) was significantly less potent than both NADA and capsaicin. The exposure of cells to NADA (0.5 and 1  $\mu$ M) resulted in desensitization of human VR1 to subsequent stimulation by 1  $\mu$ M capsaicin (from  $51.0 \pm 1.4$  to  $13.6 \pm 1.4$  and  $8.0 \pm 0.9\%$  of the effect of ionomycin, respectively,  $n = 3$ ), whereas capsaicin (0.1  $\mu$ M) desensitized the receptor to the effect of 1  $\mu$ M NADA (from  $50.0 \pm 2.5$  to  $18.0 \pm 1.2\%$ ,  $n = 3$ ). The protein kinase C activator phorbol 12,13-dibutyrate (100 nM) but not its inactive isomer  $\alpha$ -phorbol 12,13-dibutyrate significantly enhanced the potency of NADA at human VR1 ( $EC_{50}$  from  $43 \pm 6$  to  $19 \pm 3$  nM,  $P < 0.05$  by ANOVA,  $n = 3$ ), and this effect was blocked by the protein kinase C inhibitor bis-indolemaleimide (0.2  $\mu$ M, data not shown) as shown previously for capsaicin (25).

Having established that NADA is a potent agonist at recombinant rat and human VR1, we looked for this compound in rat and bovine brain. We developed ultrasensitive MS methods by using



**Fig. 3.** NADA activates peripheral VR1. (a) Effect of increasing concentrations of NADA and anandamide on intracellular  $Ca^{2+}$  in neonatal DRG neurons. \*,  $P < 0.05$  vs. NADA (t test). (b) Inhibition by VR1 antagonists of NADA (1  $\mu$ M) or capsaicin (1  $\mu$ M) enhancement of intracellular  $Ca^{2+}$  in isolated DRG neurons. \*,  $P < 0.05$  vs. vehicle (t test). (c) Effect of increasing concentrations of NADA and anandamide on the release of CGRP-LI and SP-LI from rat dorsal spinal cord slices. (d) Effect of capsazepine pretreatment, VR1 antagonism, and incubation in a  $Ca^{2+}$ -free medium on the effect of NADA on CGRP-LI and SP-LI. \*,  $P < 0.05$  vs. vehicle (t test). ND, not detectable.

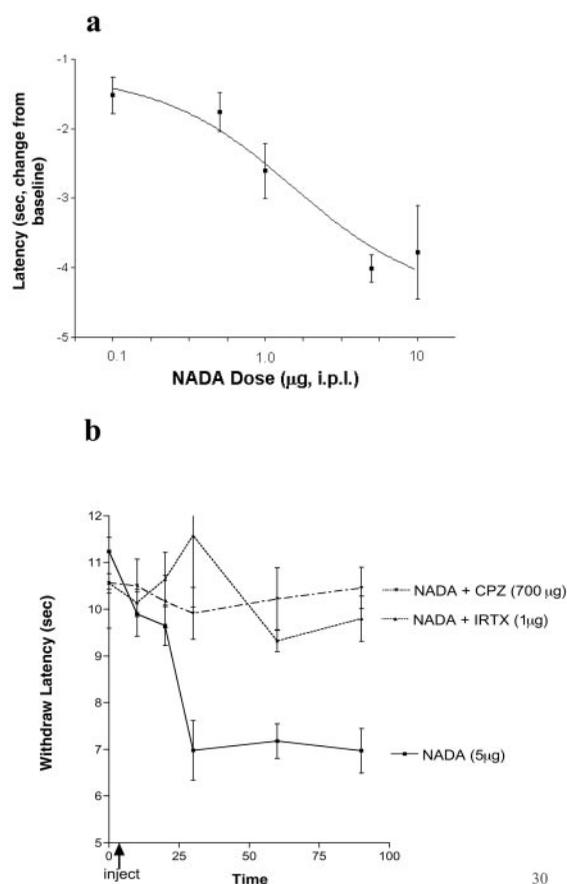
triple-quadrupole MS and quadrupole time-of-flight MS instruments. Product-ion scans in negative- and positive-ion mode of the purified bovine striatal methanol extract and synthetic NADA yielded identical mass spectra at the HPLC retention time of NADA (Fig. 2a). Quadrupole time-of-flight analysis of the material in the extract provided a high-precision estimate of the mass of the M+H ion as 440.3189 (Fig. 2c), which is within 4.3 ppm of the mass of  $C_{28}H_{42}NO_3$ , which is the chemical formula of NADA+H. Furthermore, the exact mass estimates of fragment ions provided for reconstruction of the chemical structure of NADA (Fig. 2d). To compare the chemical behavior of the extract and the standard, we treated them with acetic anhydride to form *N*-arachidonoyl-3,4-diacetoxypheylethylamine. Using multiple-reaction monitoring on a triple-quadrupole mass spectrometer, we observed coeluting peaks from the extract and the standard (Fig. 2b), which were absent in untreated extract or reagent controls. Hence, we have identified a molecule endogenous to the bovine brain that is identical to NADA in its chromatographic behavior, exact mass, fragment spectrum, and chemical behavior.

By using liquid chromatography/tandem MS under multiple-reaction monitoring mode, we found the relative amounts of NADA in various rat brain areas to be similar to the density of

VR1 (6, 8), with the highest concentrations in the striatum, hippocampus, and cerebellum (Fig. 2e). NADA was detected in small amounts also in the bovine DRG.

One possible pathway for NADA biosynthesis is the condensation of dopamine with arachidonic acid (possibly via arachidonoyl-CoA), similar to the formation of *N*-arachidonoyl-glycine (26). Another mechanism, based on the abundance of *N*-arachidonoyl amino acids in mammalian tissues (26), is via a putative *N*-arachidonoyl-tyrosine produced from the condensation of tyrosine with arachidonic acid and then converted to NADA by the same enzymes that convert tyrosine to dopamine. Evidence in support of both these routes was gained here by using rat brain homogenates incubated with [ $^3$ H]arachidonic acid and dopamine or tyrosine (Fig. 2f). In particular, the enzymatic formation of a [ $^3$ H]NADA-like compound from tyrosine was reduced when inhibiting tyrosine  $\beta$ -hydroxylase (21). More detailed studies are necessary to understand the biosynthesis of NADA in intact neurons. However, the finding that this compound is most abundant in the brain region with the highest amounts of dopamine (i.e., the striatum, Fig. 2e) supports the conclusion that NADA is produced endogenously.

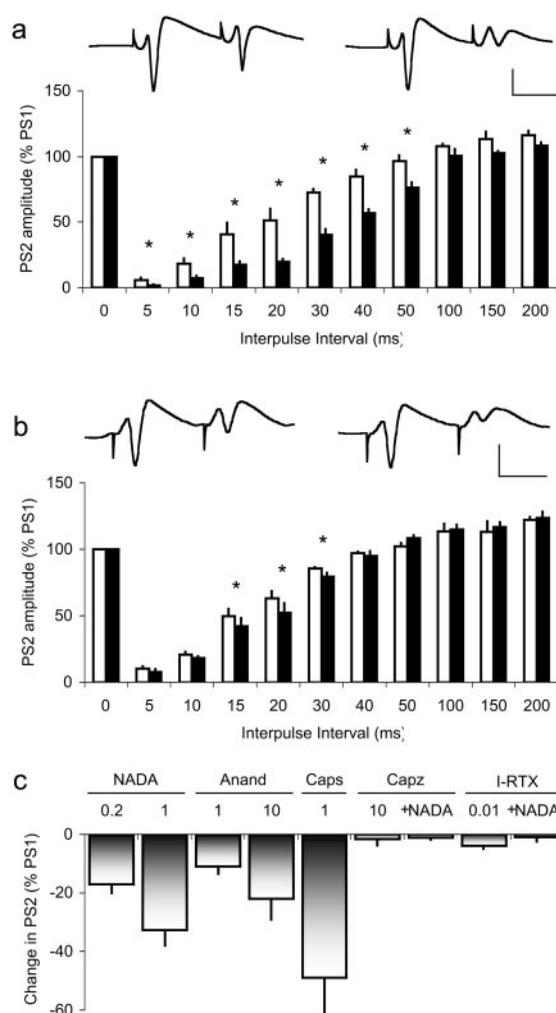
We also investigated possible mechanisms of inactivation of NADA and found that [ $^3$ H]NADA is taken up rapidly by C6 glioma



**Fig. 4.** NADA induces thermal hyperalgesia in mice via VR1. (a) The effect of NADA was dose-dependent. (b) Time response of the effect of NADA (5 μg, administered into the plantar surface of a hind paw) and its blockade by the VR1 antagonists capsazepine (CPZ) or iodo-resiniferatoxin (IRTX), administered 10 min before NADA (data are means ± SEM of  $n = 50$  for NADA alone,  $n = 19$  for NADA+CPZ, and  $n = 15$ , for NADA+IRTX).

cells via the anandamide membrane transporter (27), with apparent  $K_m$  and  $B_{max}$  being  $55 \pm 8 \mu M$  and  $1.9 \pm 0.3 \text{ nmol min}^{-1}$  per mg of protein, respectively, as compared with  $11.2 \pm 2.0 \mu M$  and  $1.7 \pm 0.3 \text{ nmol min}^{-1}$  per mg of protein for anandamide (22). However, because the VR1 binding site for ligands is intracellular (28, 29), interaction with the anandamide membrane transporter may represent either a mechanism for intracellular NADA to be taken away from VR1 or a necessary route for extracellular NADA to interact with VR1 (28). Compared with anandamide, NADA is hydrolyzed slowly by rat brain or liver fatty acid amide hydrolase (30) to dopamine and arachidonic acid ( $20.1 \pm 3.2$  and  $12.2 \pm 2.5 \text{ pmol min}^{-1}$  per mg of protein). NADA is converted also ( $2.0 \pm 0.6 \text{ pmol min}^{-1}$  per mg of protein) to the less potent 3-*O*-methyl derivative by catechol-*O*-methyl-transferase from rat liver cytosol, suggesting that methylation represents a mechanism for the partial inactivation of NADA in nervous tissues where this enzyme is abundant.

We next investigated the effect of NADA on native vanilloid receptors. Because the DRG contains the highest density of VR1 in both rat and human (6–10), we tested NADA in this tissue. The compound enhanced intracellular  $\text{Ca}^{2+}$  in cultured DRG neurons from newborn rats ( $EC_{50} = 794 \pm 95 \text{ nM}$ , Fig. 3a), this effect being attenuated by the two VR1 antagonists, capsazepine and iodo-resiniferatoxin (ref. 31; Fig. 3b). In the same assay, capsaicin and NADA were equipotent, both being approximately five times more potent than anandamide (Fig. 3a and b and data not shown). Furthermore, NADA significantly stimulated the



**Fig. 5.** NADA activates VR1 and enhances paired-pulse depression in the rat hippocampal slice. (a) Example of synaptic responses recorded from a single slice stimulated with paired pulses at an interstimulus interval of 20 ms. The response on the *Left* was recorded under control conditions, and the one on the *Right* was recorded after perfusion with 1 μM NADA for 20 min. Note no change in PS1 but a decrease in the amplitude of PS2. (Scale bar, 2 mV and 10 ms.) The histogram shows the effect of NADA over a wider range of interstimulus intervals. Open bars show control paired-pulse depression, and filled bars show that recorded after perfusion with 1 μM NADA. Bars represent mean ± SEM ( $n = 5$ ), and \* indicates a significant difference ( $P < 0.05$ , paired *t* test) from control at that specific interstimulus interval. (b) Effect of 1 μM anandamide perfused for 20 min. (Scale bar, 1 mV and 10 ms.) (c) The summary histograms represent the change in PS2 amplitude evoked by 0.2 ( $n = 3$ ) and 1 ( $n = 5$ ) μM NADA, 1 and 10 μM anandamide ( $n = 4$ ), 1 μM capsaicin ( $n = 5$ ), 10 μM capsazepine ( $n = 3$ ), 1 μM NADA in the presence of capsazepine (CAPZ,  $n = 3$ ), 10 nM iodo-resiniferatoxin (I-RTX,  $n = 3$ ), and 1 μM NADA in the presence of iodo-resiniferatoxin ( $n = 3$ ). The values shown for 1 μM capsaicin and 10 μM anandamide are from ref. 24.

release of SP-LI and CGRP-LI from slices of rat dorsal spinal cord (where the central endings of VR1-containing DRG neurons terminate) at 10–100 nM concentrations, whereas anandamide required 10-fold higher concentrations (Fig. 3c). This effect of NADA was abolished by VR1 antagonism, the lack of extracellular calcium, and pretreatment of the slices with capsaicin (Fig. 3d). The lower potency of NADA in DRG neurons (but not in the adult rat dorsal spinal cord) compared with HEK293 cells overexpressing rat VR1 can be explained by the higher degree of expression of VR1 in these cells.

The peripheral (skin) terminals of small-diameter DRG neurons also are rich in VR1 receptors, whose excitation by capsaicin,

noxious heat, and low pH evokes the burning sensation typical of inflammatory/thermal hyperalgesia, a pathological condition absent in mice with genetically deleted VR1 receptors (VR1 knock-outs; refs. 32 and 33). We found that intradermal administration of NADA in the hind paw dose- and time-dependently induced strong thermal hyperalgesia. This effect of NADA was comparable, both in efficacy and sensitivity to VR1 blockade, to those induced by carrageenan and capsaicin (Fig. 4; data not shown; refs. 15 and 32). Equimolar doses of arachidonic acid and dopamine administered alone were inactive (data not shown). Although NADA was very potent in this assay, the concentration needed to achieve half-maximal stimulation of the hyperalgesic response ( $1.5 \pm 0.3 \mu\text{g}$ , equivalent to  $\approx 3.4 \text{ nmol}$ ) was much higher than that found in DRG and skin. At first glance, this result could indicate that NADA has little or no role in sensory processing. However, higher basal levels of NADA would induce pain and desensitize VR1. Indeed, inflammatory/proalgesic lipid mediators, and eicosanoids in particular, generally do not occur in high amounts under basal conditions and are produced "on demand." Furthermore, other conditions that activate VR1 such as other endovanilloids (12–14), heat, protons, enhanced protein kinase C (4, 25), and/or phospholipase C activity (34), alone or in combination, might concur with NADA to VR1 stimulation during inflammation.

As shown above, NADA was found at the highest concentrations in two rat brain regions, the striatum and hippocampus, where significant VR1 expression has been detected previously (6–10). Although not studied exhaustively, the occurrence of VR1 receptors in the brain has been well demonstrated and may explain a variety of central nervous system actions of capsaicin in rats such as hypothermia (2), reduction of spontaneous activity (35), and modulation of hippocampal plasticity (24). To assess whether NADA also activates central VR1 receptors we choose to test its effect on this latter phenomenon, which has been characterized best from a neurochemical point of view (24). In the CA1 region of the rat hippocampus, the amplitude of PS2 evoked at a short latency after the first (PS1) is depressed, apparently because of the inhibitory feedback exerted by  $\gamma$ -aminobutyric acid released after the first pulse. This phenomenon is enhanced by capsaicin and, at higher concentrations, by anandamide in a manner sensitive to VR1 blockade. Both drugs therefore diminish the amplitude of the second PS, possibly via stimulation of  $\gamma$ -aminobutyric acid release (24). Using this paradigm of synaptic plasticity, we found that NADA, similar to capsaicin, had no effect on the amplitude of PS1 but enhanced paired-pulse depression recorded at interpulse intervals between 5 and 50 ms (by using the 20-ms interpulse interval,

1  $\mu\text{M}$  NADA reduced the amplitude of PS2 from  $52 \pm 9$  to  $20 \pm 3\%$  of PS1,  $n = 5$ ; Fig. 5a). In interleaved experiments, taking slices from the same animals, anandamide (1  $\mu\text{M}$ ) caused a much smaller enhancement of paired-pulse depression (from  $63 \pm 6$  to  $52 \pm 7\%$ ,  $n = 4$ ; Fig. 5b). NADA was approximately equipotent to capsaicin and significantly more potent than 5- to 10-fold lower doses of anandamide. Perfusion of VR1 antagonists blocked the effect of subsequently applied NADA (Fig. 5c). These data suggest that NADA might play a role in the regulation of hippocampal plasticity.

In conclusion, we have reported the identification of NADA in rat and bovine nervous tissue. NADA is the first endogenous compound identified in mammals that exhibits potency and efficacy at VR1 comparable to that of capsaicin, as predicted from the presence in its chemical structure of a catechol moiety very similar to the vanillyl moiety of potent plant-derived and synthetic VR1 agonists. Because, in addition to VR1, NADA is capable of activating CB<sub>1</sub> receptors (although at higher doses; ref. 20), future work using assays that can be performed in VR1 knockout mice is needed. Nevertheless, it is clear that NADA produces VR1-mediated effects in both central and peripheral rat neurons at doses at least 10-fold lower than those required for any of the putative endogenous VR1 agonists identified to date. Not only does NADA produce the same effects as capsaicin in the assays examined, but all its actions are blocked both by capsazepine and the recently developed, much more potent and selective VR1 antagonist, iodoresiniferatoxin (31). NADA is most abundant and is biosynthesized and inactivated in the brain, and its discovery provides a "raison d'être" to the presence of VR1 in the central nervous system. However, the physiological function of NADA as a brain endovanilloid, supported by its potent effect on hippocampal paired-pulse depression, awaits further studies.

We thank Applied Biosystems for their assistance with the quadrupole time-of-flight analysis and Ines Brandi, Alessia Ligresti, John Medeiros, and Marie Starekiewicz for assistance. S.M.H., J.F.K., T.J.P., C.J.C., J.D.M., and J.M.W. performed the isolation, identification, and tissue distribution of endogenous NADA and the characterization of pain behavior; M. Trevisani, M. Tognetto, and P.G. performed the assays of DRG neurons and spinal cord slices; A.A.-H. and S.N.D. performed the assay of hippocampal slices; and T.B., L.D.P., F.F., and V.D.M. performed the synthesis of compounds, metabolic studies, and assays of HEK293 cells. This work was supported by Ministero dell'Università e della Ricerca Scientifica e Tecnologica Grants 3933 (to V.D.M.) and 2975 (to P.G.), a grant from Associazione di Ricerca e Cura dell'Asma-Padua (to P.G.), and U.S. Public Health Service/National Institutes of Health Grants K02DA00375, DA13012, and NS33247 (to J.M.W.).

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