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Identification of a New Class of Molecules, the Arachidonyl Amino Acids, and Characterization of One Member That Inhibits Pain*

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Susan M. Huang‡, Tiziana Bisogno§, Timothy J. Petros‡, Sai Y. Chang‖, Paul A. Zavitsanos, Robert E. Zipkin‡‡, Ramachandran Sivakumar‡‡, Andrew Coop§§, Dean Y. Maeda§§, Luciano De Petrocellis¶, Sumner Burstein¶¶, Vincenzo Di Marzo§, and J. Michael Walker‡‖‖**

*From the ‡Departments of Psychology and Neuroscience, Brown University, Providence, Rhode Island 02912, §Istituto per la Chimica di Molecole di Interesse Biologico and ¶Istituto di Cibernetica-Consiglio Nazionale delle Ricerche, Endocannabinoid Research Group, Via Toiano 6, 80072 Arco Felice, Napoli, Italy, ‖Applied Biosystems, Framingham, Massachusetts 01701, **Agilent Technologies, Wilmington, Delaware 19808, ‡‡BIOMOL Research Laboratories Inc., Plymouth Meeting, Pennsylvania 19462, §§Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, Baltimore, Maryland 21201, and ¶¶Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts 01655*

In mammals, specific lipids and amino acids serve as crucial signaling molecules. In bacteria, conjugates of lipids and amino acids (referred to as lipoamino acids) have been identified and found to possess biological activity. Here, we report that mammals also produce lipoamino acids, specifically the arachidonyl amino acids. We show that the conjugate of arachidonic acid and glycine (*N*-arachidonylglycine (NAGly)) is present in bovine and rat brain as well as other tissues and that it suppresses tonic inflammatory pain. The biosynthesis of NAGly and its degradation by the enzyme fatty acid amide hydrolase can be observed in rat brain tissue. In addition to NAGly, bovine brain produces at least two other arachidonyl amino acids: *N*-arachidonyl γ-aminobutyric acid (NAGABA) and *N*-arachidonylalanine. Like NAGly, NAGABA inhibits pain. These findings open the door to the identification of other members of this new class of biomolecules, which may be integral to pain regulation and a variety of functions in mammals.

Molecules found in bacteria that consist of a lipid moiety conjugated to an amino acid have been termed lipoamino acids (1–3). Burstein *et al.* (4) found that the lipoamino acid *N*-arachidonylglycine (NAGly)[1] causes hot plate analgesia in mice, indicating its possible biological relevance in mammals. NAGly was first synthesized (5) as a structural analog of the endogenous cannabinoid anandamide (6), and it was found to lack affinity for the cannabinoid CB1 receptor. We hypothesized that NAGly may be produced by mammalian tissues because it is composed of the naturally occurring compounds glycine and arachidonic acid. Herein we show that at least three arachidonyl amino acids are natural constituents in mammalian brain: NAGly, *N*-arachidonyl γ-aminobutyric acid (NAGABA), and *N*-arachidonylalanine (NAAla). One member of this group, NAGly, is characterized in detail here. It is synthesized *in situ* in rat brain tissue from the precursors arachidonic acid and glycine, and it is hydrolyzed by the enzyme fatty acid amide hydrolase (FAAH). NAGly is widely distributed among mammalian tissues, implying multiple functions. One possible physiological function of NAGly is pain suppression, indicated by its marked suppression of formalin-induced pain behavior in rats, confirming a previous report of analgesic activity in mice (4).

EXPERIMENTAL PROCEDURES

Tissue Extraction and Purification—The procedure comprised a liquid-liquid extraction modified from that described by Folch *et al.* (7) followed by a series of solid-phase separations. Fresh bovine brain and rat organs were homogenized in the methanol fraction of 20 volumes of 2:1 chloroform:methanol and centrifuged for 15 min at 31,000 × *g* at 4 °C. Chloroform was then added to the supernatant. NaCl (0.2 volume, 0.73%) was mixed with the crude homogenate, and the solution was allowed to separate overnight at 4 °C or centrifuged at 1,000 × *g* for 15 min. The upper phase was discarded and the interphase washed twice. The lower phase was then applied to diethylaminopropyl silica-based solid-phase extraction columns (DEA, Varian, Harbor City, CA) without prior column conditioning. The columns were washed with chloroform, methanol, 0.1% ammonium acetate in methanol and eluted with 0.5% ammonium acetate in methanol. Water (1.2 volumes) was added to the eluent, and the solution was loaded onto preconditioned Empore C18 particle loaded membrane cartridges (3M, St. Paul, MN). The cartridges were washed with water, 60% methanol and eluted with 65% methanol. Samples were evaporated in a SpeedVac (Savant Instruments, Halbrook, NY) and reconstituted prior to analysis. *N*-[$^2H_8$]Arachidonylglycine[2] was used as an internal standard in the tissue distribution study.

Ion Trap LC/MS/MS Analysis—One set of experiments for structural elucidation of NAGly in bovine brain extract was conducted with HP1100 series LC/Agilent Ion Trap. Samples were chromatographed on a 50-mm Zorbax Eclipse C18 column (2.1 mm internal diameter, 0.4 ml/min, 40 °C) with a linear gradient from 0% 10 mM ammonium acetate to 95% acetonitrile for the first 3 min, maintained at 95% for 2 min, and then back to 0% in 0.5 min. The Ion Trap was set to electrospray positive ion mode, monitoring for daughter ions produced from *m/z* 362.3 (MH^+ ion of NAGly) at the retention time of NAGly standard.

Quadrupole-TOF LC/MS/MS Analysis—Exact mass determination and structural information of NAGly, NAGABA, and NAAla in bovine brain extract were accomplished with a quadrupole-time-of-flight LC/MS/MS. Brain extract was chromatographed on a C18 column (2 × 20 mm), and the eluent was analyzed by Pulsar (qq-TOF instrument, Applied Biosystems-MDS Sciex) for mass measurements of MH^+ and

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‖‖ To whom correspondence should be addressed: The Alan M. Schrier Research Laboratory, Brown University, 89 Waterman St., P. O. Box 1853, Providence, RI 02912. Tel.: 401-863-2048; Fax: 401-863-1300; E-mail: J_Walker@brown.edu.

[1] The abbreviations used are: NAGly, *N*-arachidonylglycine; NAGABA, *N*-arachidonyl γ-aminobutyric acid; FAAH, fatty acid amide hydrolase; LC/MS, liquid chromatography/mass spectrometry; TOF, time-of-flight.

[2] Synthesized by R. E. Zipkin and R. Sivakumar.

product ions. The instrument was operated at a resolution of 9,000 (full width at half height at *m/z* 800). Mass measurements were the averages of at least 10 consecutive scans.

Analysis of Methylated N-Arachidonylglycine—For the production of methyl esters, a solution of diazomethane in ether was prepared from Diazald (Aldrich) using the apparatus and conditions described in the literature (8). This was added in excess to an ether solution of the sample to be methylated and kept at room temperature for 1 h. One drop of glacial acetic acid was then added to consume excess diazomethane. The product was then purified by silica gel tlc and its identity confirmed by LC/MS as described.

LC/MS Analysis and Quantitation—Analysis of NAGly levels was conducted with a Hewlett Packard (Palo Alto, CA) 1100 series LC/MS. Samples were chromatographed on a 100-mm Zorbax Eclipse XDB-C18 reversed phase HPLC (4.6-mm internal diameter, 1 ml/min) column with isocratic 85% methanol containing 1 mM ammonium acetate and 0.05% acetic acid. MS conditions were established using direct flow injection analysis of synthetic *N*-arachidonylglycine: APCI positive mode, fragmentor 80 V; vaporizer 500 °C; drying gas 150 °C; drying gas flow 7 liters/min; nebulizer 0.69 bar, corona 7 μA. For quantitation, the area under the peak at the appropriate retention time and *m/z* was obtained. The amount of compound was then extrapolated from a calibration curve and corrected based on extraction efficiency.

Biosynthesis of N-Arachidonylglycine—1, 10, or 100 μM [$^2H_8$]arachidonic acid (Cayman Chemicals, Ann Arbor, MI) and 2 mM [$^2H_5$]glycine (ICON, Mt. Marion, NY) (approximate free concentration in brain) was incubated in a rat P_2 membrane preparation (2.5 volumes brain weight, 407 ± 46 μg of protein/tube, 0.5 ml 50 mM Tris-HCl, 1 mM EDTA, pH 7.4) for 30 min at 37 °C. Buffer, boiled membrane, and non-incubated conditions were also tested. *N*-[$^2H_8$]Arachidonylglycine[2] was used as a batch internal standard to track extraction efficiency alongside the experimental conditions. Following incubation, the preparation was extracted via the procedure described above. Samples were analyzed via LC/MS. In some instances, samples were evaporated and reconstituted prior to analysis.

FAAH Assays—The effects of NAGly and two homologues, palmitoylglycine and linolenoylglycine, on the enzymatic hydrolysis of [^{14}C]anandamide (9 μM, 5.5mCi/mmol)[3] by membranes from cultured cells were studied as described previously (Refs. 9 and 10 and references cited therein). N18TG2 and RBL-2H3 cell membranes were incubated with increasing concentrations of compounds (50 mM Tris-HCl, pH 9, 30 min, 37 °C) (9, 10). The mixture was then extracted with 2 volumes of 2:1 chloroform:methanol, and the aqueous phase was measured for [^{14}C]ethanolamine by scintillation counting. To calculate the Lineweaver-Burk profile of FAAH inhibition by NAGly, the effect of 10 μM NAGly on the hydrolysis of increasing concentrations of [^{14}C]anandamide to [^{14}C]ethanolamine was also studied.

Hydrolysis of NAGly—To assess its enzymatic stability, NAGly (200 μg/ml) was incubated with rat brain whole homogenates (2 mg protein/ml, 50 mM Tris-HCl, pH 9, 37 °C, 30 min). Following incubation, the homogenates were extracted with 3 volumes of 2:1 chloroform:methanol, and the organic phase was lyophilized and analyzed by tlc carried out on analytical silica plates (Merck) developed with chloroform/methanol/ammonia (95:5:1 by volume). The formation of arachidonic acid from NAGly was visualized as a band with R_F = 0.4 (where R_F is relative migration) after exposure to iodine vapors. Control incubations were carried out with boiled rat brain homogenates or with homogenates incubated with [^{14}C]anandamide.

Assays of Cellular Uptake of Anandamide—The effect of NAGly on the uptake of anandamide by RBL-2H3 cells was studied with a modification of the method described previously (10), analogous to the protocol described in Hillard *et al.* (11) except for the use of a higher concentration (3.6 μM) of [^{14}C]anandamide. Residual [^{14}C]anandamide (measured by scintillation counting) in the incubation media after extraction with 2:1 chloroform:methanol was used as a measure of the anandamide taken up by cells.

Assays of Cytosolic Ca^{2+} Concentration—The effect of NAGly on the cytosolic Ca^{2+} concentration in human embryonic kidney cells transfected with the human VR1 vanilloid receptor was determined using Fluo-3, a selective intracellular fluorescent probe for Ca^{2+}. One day prior to experiments, cells cultured as described in Smart *et al.* (12) were transferred into six-well dishes coated with poly-L-lysine (Sigma) and grown in the culture medium mentioned above. On the day of the experiment, cells (50–60,000/well) were loaded with 4 μM Fluo-3 methyl ester for 2 h at 25 °C (Molecular Probes) in Me_2SO containing 0.04% Pluronic. After the loading, cells were washed with Tyrode (pH 7.4) and trypsinized to be suspended in the cuvette of the fluorescence detector (PerkinElmer LS50B) under continuous stirring. Cell fluorescence was measured (λ_{ex} = 488 nm, λ_{em} = 540 nm) before and after the addition of NAGly at various concentrations. The effect was compared with that observed with 4 μM ionomycin.

Formalin Test—The rat formalin test (13) was used as a behavior measure of pain. Rats were acclimatized to human handling and the testing chamber for at least 2 days prior to testing. Rats received injections of formalin (dissolved in mono- and dibasic phosphate saline, pH 7.4) in the left hind paw with vehicle (Me_2SO) or drug in a 90:10 ratio (100 μl, 4.5% formalin, subcutaneously, intraplantar), and the time spent licking and lifting the injected paw over the subsequent 60 min period was recorded at 5-min intervals. The summed scores of phase 1 (0–10 min) and phase 2 (10–60 min) for each group was analyzed by analysis of variance followed by Dunnet's *post hoc* comparison test (BMDP, Los Angeles, CA).

RESULTS

The identification of NAGly in the bovine brain extract was accomplished by a variety of LC/MS and LC/MS/MS approaches. Ion trap mass spectrometric analysis of the brain extract revealed that the column retention time and fragmentation pattern of the molecular ion 362.3 is identical to that of the synthetic NAGly standard, showing prominent daughter ions at *m/z* 287.2 and 269.2 (Fig. 1, *a* and *b*). Quadrupole-time-of-flight mass spectrometry yielded a mass estimate of 362.2687, which is −0.8 ppm of the expected mass of NAGly MH^+ (362.2690) with an elemental composition of $C_{22}H_{36}NO_3$ for the MH^+ ion. The loss of the amino acid moiety and water produced the fragments *m/z* 287 and 269. Further losses of lipid moieties produced *m/z* 245 and 203 (Table I, top). The exact mass measurements of the MH^+ and product ions greatly enhanced the confidence level of structural assignments, leading to reconstruction of the molecule NAGly as the material in the brain extract (Fig. 1*c* and Table I, top). In addition to the identification of the compound in its native form, we performed chemical methylation on the brain extract and the synthetic standard with diazomethane. LC-MS analysis of the treated standard and the extract revealed coeluting peaks at the expected mass of NAGly-methyl ester MH^+ (*m/z* 376.3, Fig. 1*d*). The results indicate that both the synthetic standard and the material in the brain extract underwent the same reaction and were converted to NAGly-methyl ester. Hence, the constituent we isolated from bovine brain has the elemental composition and the structural components that constitute NAGly and exhibits the same chemical properties as synthetic NAGly, thus indicating that NAGly is a naturally occurring molecule in the brain.

The identification of endogenous NAGly led us to postulate the existence of other arachidonyl amino acids in mammalian brain. Using the extraction procedure described above, we found that at least two other such molecules are present in the bovine brain. Quadrupole-time-of-flight mass spectrometry was employed to aid structural elucidation. Exact masses were determined to within 2.6 ppm of the parent MH^+ ions, accompanied by a spectrum of product ions reflecting successive losses of the amino acid, water, and lipid moieties in a manner similar to that observed for NAGly, leading to a reconstruction of the structures of two additional arachidonyl amino acids in bovine brain extract, *N*-arachidonyl γ-aminobutyric acid and *N*-arachidonylalanine (Table I, middle and bottom parts).

The presence of NAGly, NAGABA, and NAAla in the brain necessitates pathway(s) for their biosynthesis and degradation. We focused on NAGly for the characterization. To address the question of biosynthesis, we used rat brain P_2 membranes and deuterated precursors (arachidonic acid and glycine) to detect a direct coupling pathway and to facilitate detection by mass spectrometry. Deuterated NAGly was not detected when buffer

[3] Synthesized by T. Bisogno and V. Di Marzo.

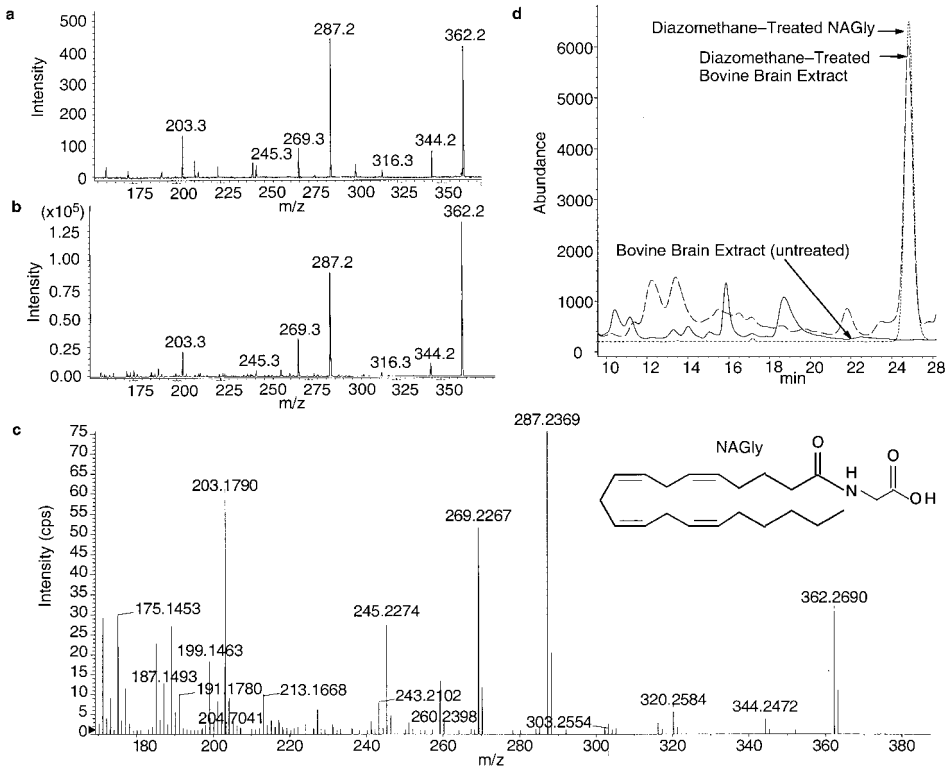

FIG. 1. **Structural elucidation of a brain constituent as NAGly.** Identical fragmentation patterns are revealed from synthetic NAGly (*a*) and the constituent found in bovine brain extract (*b*) via ion trap LC/MS. The prominent parent ion at *m/z* 362.3 (MH^+ of NAGly) and product ions at *m/z* 287.2, 269.3, and 203.3 are observed for both the standard and the extract at the NAGly retention time, 4.7 min. *c*, exact mass determination of the compound in brain extract. The mass spectrum shows the collision-induced fragments (product ion scan) from bovine brain extract via Quadrupole-TOF LC/MS. The high mass resolution of the molecular ion and the fragment ions greatly enhances the precision of structural assignment (see Table I, top). The *inset* shows the structure of NAGly. *d*, co-elution of diazomethane-treated synthetic NAGly and brain extract via LC/MS in selected ion monitoring mode at *m/z* 376.2 (MH^+ of NAGly methyl ester). No such material was observed from the untreated extract.

TABLE I

Mass measurement of MH^+ and the product ions and structural assignments from bovine brain extract conducted with Quadrupole-TOF LC/MS/MS

Common fragments[a]	Proposed formulae	Comments
m/z (ppm)		
Putative *N*-arachidonyl glycine		
362.2687 (−0.8)	$C_{22}H_{36}NO_3$	MH^+
287.2368 (−0.5)	$C_{20}H_{31}O$	$MH^+ - C_2H_5NO_2$ (Gly)
269.2266 (0.8)	$C_{20}H_{29}$	$MH^+ - C_2H_5NO_2$ (Gly) $- H_2O$
245.2273 (3.8)	$C_{18}H_{29}$	$MH^+ - C_4H_7NO_3$
203.1788 (−3.1)	$C_{15}H_{23}$	$MH^+ - C_7H_{13}NO_3$
Putative *N*-arachidonyl γ-aminobutyric acid		
390.3012 (2.4)	$C_{24}H_{40}NO_3$	MH^+
287.2382 (4.4)	$C_{20}H_{31}O$	$MH^+ - C_4H_9NO_2$ (GABA)
269.2279 (5.7)	$C_{20}H_{29}$	$MH^+ - C_4H_9NO_2$ (GABA) $- H_2O$
245.2269 (2.1)	$C_{18}H_{29}$	$MH^+ - C_6H_{11}NO_3$
203.1795 (0.4)	$C_{15}H_{23}$	$MH^+ - C_9H_{17}NO_3$
Putative *N*-arachidonylalanine		
376.2856 (2.6)	$C_{23}H_{38}NO_3$	MH^+
287.2369 (−0.2)	$C_{20}H_{31}O$	$MH^+ - C_3H_7NO_2$ (Ala)
269.2238 (−9.6)	$C_{20}H_{29}$	$MH^+ - C_3H_7NO_2$ (Ala) $- H_2O$
245.2[b]	$C_{18}H_{29}$	$MH^+ - C_5H_9NO_3$
203.1[b]	$C_{15}H_{23}$	$MH^+ - C_8H_{15}NO_3$

[a] Mass measurements and errors (ppm) of at least 10 consecutive scan averages.
[b] Due to low signal level, exact mass measurements were not performed.

or boiled membranes were incubated with the precursors or when membranes were incubated without the precursors (Fig. 2). However, when fresh rat brain membranes were incubated with [$^2H_8$]arachidonic acid and [$^2H_5$]glycine in Tris-HCl buffer at 37 °C for 30 min, deuterated NAGly was produced (Fig. 2 and *inset*). This was detectable at masses corresponding to the molecular ions ranging from [$^2H_8$]NAGly to [$^2H_{12}$]NAGly (the varying degree of incorporation of deuterium expected because of proton exchange with the aqueous buffer and the tissue). Maximal responses were observed at the molecular ion of [$^2H_{10}$]- and [$^2H_9$]NAGly. The presence of deuterated NAGly following incubation indicates biosynthesis of NAGly from arachidonic acid and glycine via an enzymatic process.

To address the question of degradation of NAGly, we tested its affinity for the enzyme fatty acid amide hydrolase, which acts upon substrates of similar structure such as anandamide and oleamide (14, 15). When coincubated with [^{14}C]anandamide, NAGly potently and competitively inhibited its hydrolysis

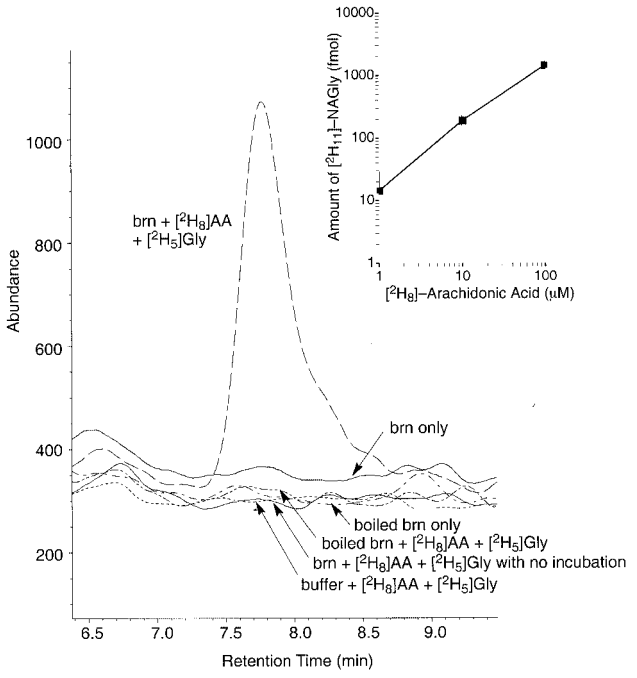

FIG. 2. **Biosynthesis of NAGly in brain tissue.** Deuterated NAGly was detected when [$^2H_8$]arachidonic acid (*AA*) and [$^2H_5$]glycine (*Gly*) were incubated with rat brain (*brn*) P_2 membrane preparation (37 °C, pH 7.4, 30 min, 50 mM Tris-HCl, at least six replications of each experiment). The chromatogram illustrates the amount of deuterated NAGly measured using selected ion monitoring at *m/z* 372.3 (MH^+ of [$^2H_{10}$]NAGly) following incubation of 100 μM [$^2H_8$]arachidonic acid and 2 mM [$^2H_5$]Gly. The *inset* indicates the biosynthesis of [$^2H_{11}$]NAGly at various concentrations of [$^2H_8$]arachidonic acid (1, 10, 100 μM, $n = 6$).

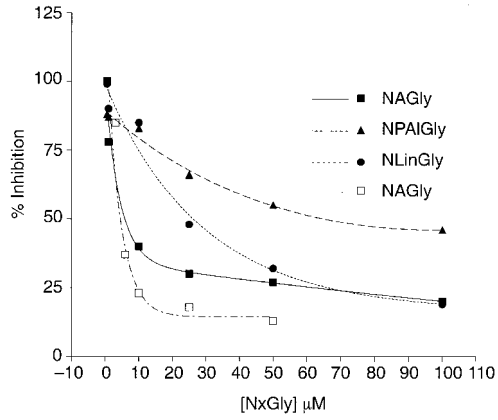

FIG. 3. **NAGly inhibition of anandamide breakdown by fatty acid amide hydrolyase.** Effect of NAGly (*closed squares*) and two homologues, palmitoylglycine (*NPalGly*) and linolenoylglycine (*NLinGly*), on the hydrolysis of [^{14}C]anandamide by N18TG2 cell membranes. A similar effect of NAGly is also found in RBL-2H3 membranes (*open squares*). Data are the means of $n = 3$. S.E. were not shown for the sake of clarity and were never higher then 5% of the means.

by N18TG2 and RBL-2H3 cell membranes/preparations that contain FAAH (IC_{50} 7.0 and 4.1 μM; Fig. 3). The inhibition was competitive according to Lineweaver-Burk analysis. Ten μM NAGly decreased the K_m of the enzyme (from 13 ± 1 to 9 ± 1 μM) without decreasing its V_{max} (from 2.2 ± 0.7 to 2.1 ± 0.5nmol/min/mg protein). The rank potency of inhibition obtained with NAGly and the two analogues (palmitoylglycine and linolenoylglycine) are identical to those observed with anandamide, palmitoylethanolamide, and linolenoylethanolamide, thus strengthening the hypothesis that NAGly is acting on FAAH. When NAGly was incubated with rat crude membrane homogenate, it was readily hydrolyzed to arachidonic acid. By densitometry scanning of tlc plates, it was estimated that after 30 min at 37 °C, about 15% of NAGly had been hydrolyzed (data not shown). The results suggest that one likely mechanism of inactivation of NAGly in mammalian tissues is through enzymatic degradation by FAAH. We also investigated whether NAGly could be recognized by the membrane transporter that facilitates anandamide uptake by cells (16). NAGly, up to a 50 μM concentration, failed to inhibit the transport of [^{14}C]anandamide into RBL-2H3 cells, which contain active anandamide transporters. This latter finding suggests that if NAGly is transported into cells, it does so through a mechanism distinct from the anandamide transporters.

The similarity between the structures of NAGly and anandamide and their common mode of degradation led us to wonder whether they share other molecular targets. The lack of affinity of NAGly for cannabinoid receptors has been mentioned above. However, because anandamide also acts as a full agonist at the rat and human vanilloid VR1 receptors (12, 17), we tested the action of NAGly at the human VR1 receptor. NAGly was inactive (<5% at 10 μM) in inducing VR1-mediated Ca^{2+} influx in VR1-overexpressing human embryonic kidney cells.

Although we initially isolated NAGly from the brain, subsequent studies revealed varying levels in different tissues in the rat (Fig. 4). The important role of arachidonic acid-derived products (*e.g.* prostanoids, leukotrienes, and thromboxanes) in inflammation and pain, together with the relatively high levels of NAGly in the skin, spinal cord, and brain, suggested that NAGly may serve as a modulator of pain and inflammation.

The above observations on NAGly along with a previous report (4) of its antinociceptive actions led us to study the effects of NAGly on pain behavior using the formalin test. Rats received intraplantar injections of formalin in the hind paw, and pain behavior consisting of lifting and licking of the injected paw was observed. Formalin injection elicited a robust two-phase pain response, consisting of a brief first phase (acute pain), a transient remittance of pain behavior, and a prolonged second phase (tonic pain, Fig. 5, *inset*). Neither glycine nor arachidonic acid (275 nmol) suppressed pain when coinjected with formalin, and NAGly had no effect on the first phase of the formalin response. However, in the second phase, 275 nmol of NAGly markedly suppressed the pain response elicited by formalin. No behavioral abnormality was observed in the drug-treated animals.

DISCUSSION

A new family of bioactive molecules was identified in mammalian brain, the arachidonyl amino acids. Using a variety of approaches, three members of this group were identified in this study: NAGly, NAGABA, and NAAla. NAGly was chosen as a prototypical molecule for detailed characterization because of its structural similarity to other arachidonyl amino acids and previous indications of its bioactivity (4). There is a high likelihood that members of this family, such as NAGABA and NAAla, share similar mechanisms of action, in particular with respect to the biosynthetic and degradatory pathways.

NAGly was found to be synthesized from precursors arachidonic acid and glycine in rat brain tissues via an enzymatic process. Although the details of NAGly biosynthesis require further elucidation, one mechanism involving enzymes of known activities can be proposed. Arachidonic acid can be converted to arachidonyl-coenzyme A (CoA) by a class of enzymes, acyl-CoA synthetase. To date, one such enzyme with a distinct specificity for arachidonic acid, arachidonyl-CoA synthetase, has been found in the brain, platelets, and aorta (18–20). A second family of enzymes, acyl-coenzyme A:glycine *N*-acyltransferases, can conjugate glycine to various aliphatic and aromatic acyl-CoAs (21–26). These enzymes have varying sub-

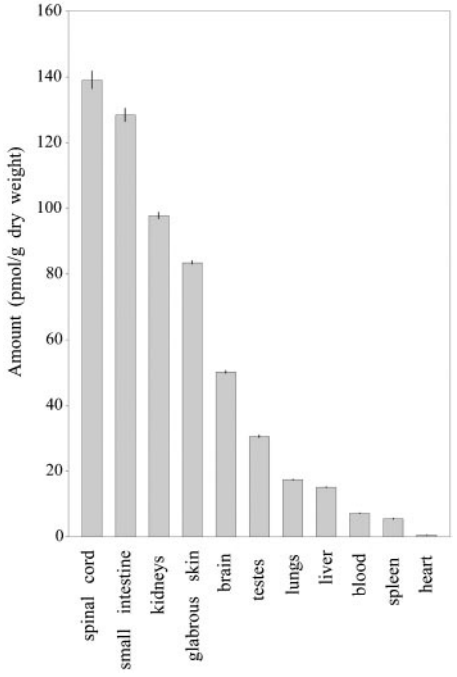

FIG. 4. **NAGly is found in a variety of tissues in the rat.** Fresh rat organs were extracted and analyzed for NAGly by LC/MS analysis using selected ion monitoring of the mass of the principal MH^+ ion, *m/z* 362.3, and the characteristic fragment 287.3 (arachidonic acid-H_2O). Tissue dry weights were estimated from the ratio of lyophilized to wet weight of each type of tissue.

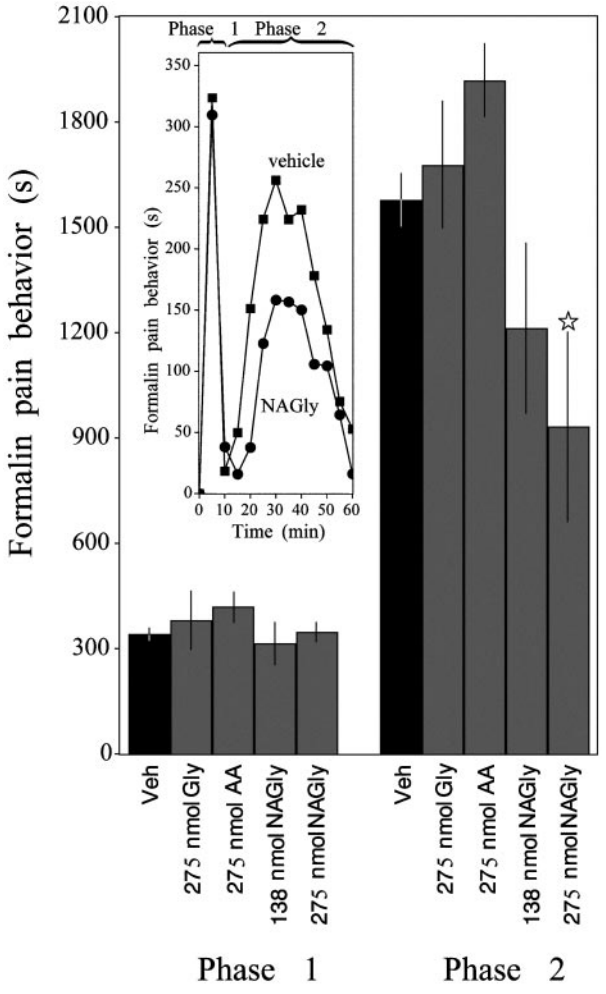

FIG. 5. **NAGly suppression of formalin-induced pain behavior.** Peripheral administration of 275 nmol of NAGly in the hind paw significantly suppressed phase 2 response of formalin-induced pain behavior in the rat ($p < 0.05$). NAGly did not have an effect on the phase 1 response. Mean (±S.E.) pain behavior score determined as the time animals spent licking plus the time spent lifting the formalin-injected paw. 0–10 min comprises phase 1, whereas 10–60 min comprises phase 2 (*inset*). Equimolar amounts of arachidonic acid (*AA*) or Gly were tested to check for possible contributions from breakdown products. *, $p < 0.05$; significant difference from the vehicle (*Veh*), glycine, and arachidonic acid groups. *Inset*, mean formalin pain behavior over the 1-h period for animals that received vehicle (*squares*) and those that received 275 nmol of NAGly (*circles*).

strate specificity depending on the hydrocarbon structure of the individual acyl-CoA. The existence of specific arachidonyl-CoA synthetase and the specialized role of arachidonic acid in mammalian physiology support the notion that the biosynthesis of NAGly may occur via actions of arachidonyl-CoA synthetase and an enzyme of the acyl-coA:glycine *N*-acyltransferase family.

NAGly shares one of the molecular mechanisms of anandamide, namely degradation by the enzyme FAAH. In fact, NAGly exhibits higher potency for FAAH inhibition than anandamide. These results, along with NAGly's lack of affinity for the cannabinoid CB1 receptor, the vanilloid VR1 receptor, and the anandamide transporter, support the notion that despite its structural similarity to the endogenous cannabinoid anandamide, NAGly likely functions via molecular and cellular pathways distinct from anandamide and other lipid mediators.

NAGly was effective in suppressing phase 2 (tonic pain phase) of formalin-induced pain behavior. A combination of mechanisms appears to contribute to the phase 2 pain behavior. Both persistent peripheral nociceptor discharge and the ensuing central sensitization in the spinal cord are important in the initiation and maintenance of the spontaneous pain, allodynia and hyperalgesia (27–30). This tonic pain phase has been likened to persistent postoperative pain (reviewed in Ref. 31). The efficacy of peripherally administered NAGly in inhibiting phase 2 pain behavior suggests that NAGly likely suppressed the formalin-induced hyperactivity in nociceptive afferents either directly, on the nerve, or indirectly by modulating their immediate interstitial environment. Because either action would minimize central sensitization leading to reduced pain following tissue injury, the suppression of formalin-induced pain by NAGly may have relevance to postoperative and chronic pain states.

Along with the ability of NAGly ability to suppress pain, a previous report showed that NAGABA has analgesic properties in mice, its potency being equivalent to that of delta-9-tetrahydrocannabinol (Δ^9-THC) (4). It also produces motor dysfunction in this species (4). These findings support the notion that NAGly, NAGABA, and NAAla are members of a family of *N*-arachidonyl amino acids in mammals that may have overlapping functions such as pain modulation.

In addition to pain, this new group of compounds are likely to be active in other domains of physiology. This notion is supported by the wide distribution of NAGly in rat tissues. Among the tissues analyzed, relatively high levels of NAGly were present in the spinal cord, small intestine, kidneys, skin, and brain, whereas more modest levels were found in testes, lungs, and liver. The varying levels of NAGly in different organs likely indicate its involvement in additional physiological functions besides pain regulation. Furthermore, the dual effect NAGABA on pain and motor function (4) suggests that the family of arachidonyl amino acids discovered here likely has relevance in multiple physiological systems.

In summary, our results show that *N*-arachidonylglycine is present in mammals, synthesized *in situ* from arachidonic acid

and glycine, and degraded by fatty acid amide hydrolase. Consistent with its high levels in skin and neural tissues, NAGly is capable of suppressing pain via a peripheral action, suggesting that it may serve endogenously to regulate pain. The identification of endogenous NAGly, NAGABA, and NAAla points to a new class of biomolecules that likely serve a variety of regulatory functions in brain and other tissues.

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Identification of a New Class of Molecules, the Arachidonyl Amino Acids, and Characterization of One Member That Inhibits Pain

Susan M. Huang, Tiziana Bisogno, Timothy J. Petros, Sai Y. Chang, Paul A. Zavitsanos, Robert E. Zipkin, Ramachandran Sivakumar, Andrew Coop, Dean Y. Maeda, Luciano De Petrocellis, Sumner Burstein, Vincenzo Di Marzo and J. Michael Walker

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