ELSEVIER

Biochemical Pharmacology 64 (2002) 1147–1150

Biochemical Pharmacology

Short Communication

Regulation of anandamide tissue levels by *N*-arachidonylglycine

Sumner H. Burstein[a,b,*], Susan M. Huang[c], Timothy J. Petros[c], Ronald G. Rossetti[b], J Michael Walker[c], Robert B. Zurier[b]

[a]*Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Room 803, New Research Building, 264 Plantation Street, Worcester, MA 01605, USA*

[b]*Department of Medicine, Division of Rheumatology, University of Massachusetts Medical School, Worcester, MA 01605, USA*

[c]*Departments of Psychology and Neuroscience, Brown University, 89 Waterman Street, Providence, RI 02912, USA*

Received 12 February 2002; accepted 30 April 2002

Abstract

N-Arachidonylglycine (NAGly), the carboxylic analog of the endocannabinoid anandamide, occurs in rat and bovine brain as well as in peripheral sites and shows activity against tonic, formalin-induced pain. It was also observed, using cell membrane preparations, that it inhibits the hydrolytic activity of fatty acid amide hydrolase (FAAH) on anandamide (*N*-arachidonylethanolamide). These data suggested that it may serve as an endogenous regulator of tissue anandamide concentrations. In this report, we show findings derived from mass spectrometric analyses, indicating that blood levels of anandamide in rats given 10 mg/kg p.o. of NAGly were increased significantly by more than 9-fold when compared with vehicle-treated controls. *In vitro* evidence in RAW 264.7 cells using a deuterium-labeled NAGly demonstrated that it was not a precursor or source of arachidonic acid for the observed 50% rise in anandamide levels, suggesting that the increase was due to some effect other than increased biosynthesis of anandamide. Moreover, the findings presented here suggest that NAGly can serve as a model for the design of agents to provide pharmacological control of tissue anandamide concentrations.

Keywords: Anandamide; *N*-Arachidonylglycine; RAW cells; Rats; Mass spectrometry

1. Introduction

NAGly (Fig. 1), an endogenous substance found in rat brain and other sites [1], occurs in amounts greater than the closely related endocannabinoid anandamide (*N*-arachidonylethanolamide). Earlier reports [2] suggested that NAGly may have analgesic properties similar to those of anandamide [3,4] but is inactive in assays for psychotropic action such as the "ring test" [5]. The latter finding was in agreement with a report showing a lack of binding by NAGly to the cannabinoid receptor CB1 [6]. This activity profile is reminiscent of that observed for the naturally occurring cannabinoid acids [7], which exhibit analgesic effects but are inactive in the ring test. NAGly also showed substantial potency ($EC_{50} = 4-7$ μM) in reducing the *in vitro* activity of FAAH [1,8], the enzyme primarily responsible for the degradation of anandamide to arachidonic acid and ethanolamine under physiological conditions [9–11]. However, it has little effect on anandamide transport or on the VR1 vanilloid receptor [1]. This suggests, as one of several possibilities, that NAGly may act as an endogenous regulator of tissue anandamide concentrations by virtue of its ability to inhibit FAAH and its presence in a number of tissue sites *in vivo* [1]. NAGly also showed *in vivo* anti-inflammatory activity in the mouse paw edema assay (Burstein SH and Pearson W, unpublished data).

The biological origin of NAGly is not well understood; however, two possible biosynthetic pathways have been investigated, and data supporting the existence of each have been reported [1,12]. Burstein *et al.* [12], using anandamide radiolabeled in the ethanolamine moiety, showed that Chang hepatocytes incubated with this precursor produced a radiolabeled substance that chromatographically co-migrated with NAGly in several TLC systems. This suggested the possibility that NAGly may, under some conditions, be generated by an oxidative metabolism of anandamide. A second pathway involving the condensation of arachidonyl CoA with glycine was proposed by Huang *et al.* [1] in which the process is

* Corresponding author. Tel.: +1-508-856-2850; fax: +1-508-856-2003.
E-mail address: sumner.burstein@umassmed.edu (S.H. Burstein).
Abbreviations: FAAH, fatty acid amide hydrolase; and NAGly, *N*-arachidonylglycine.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)01301-1

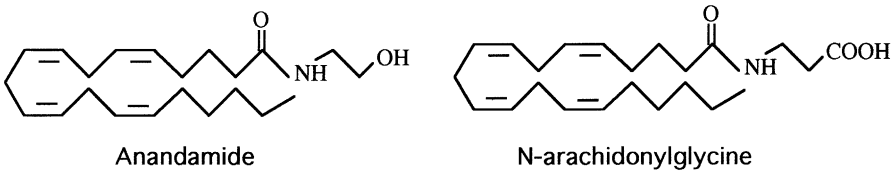

Fig. 1. Structures of the endocannabinoid anandamide and its endogenous analog *N*-arachidonylglycine (NAGly).

mediated by a subcellular rat brain preparation. To support this suggestion, they used deuterium-labeled precursors and demonstrated the synthesis of deuterated NAGly by mass spectrometric analysis. As is often the case in biosyntheses, each pathway may operate under a specific set of physiological circumstances.

We sought to determine in this study whether the *in vivo* administration of NAGly would increase circulating blood levels of anandamide that would, in turn, reflect an elevation of cellular concentrations of anandamide. This might help explain the analgesic [1] and anti-inflammatory actions of NAGly (Burstein SH and Pearson W, unpublished data) since anandamide is known to exhibit both actions in experimental models [3,4,13]. The results obtained from these studies are reported below.

2. Materials and methods

2.1. Materials

RAW 264.7 murine monocyte cells were prepared from stocks supplied and maintained by the University tissue culture facility. Minimum essential medium (MEM) was purchased from ICN. Fetal bovine serum and penicillin–streptomycin solution were obtained from GIBCO BRL. Deuterated arachidonic acid (d_8-arachidonic acid) was obtained from the Cayman Chemical Co. Bovine serum albumin was obtained from Sigma, and Sep-Pak Plus C18 cartridges were purchased from the Waters Corp. TLC plates were obtained from EM Science.

2.2. Synthesis of deuterated N-arachidonylglycine (d_8-NAGly)

To a solution of *N*-hydroxysuccinimide (4 mg) in 5 mL of ethyl acetate, a solution of 10 mg d_8-arachidonic acid (5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic-5,6,8,9,11,12,14,15-d_8 acid) (>98 atom% D) was added followed by 9 mg of dicyclohexylcarbodiimide. The mixture was allowed to react for 24 hr at room temperature at which time 10 mg of glycine in a mixture of dioxane–KOH–$NaHCO_3$ (2 mL) was added and reacted for a further 48 hr at 4°. The mixture was then acidified to pH 3 with HCl, extracted with ethyl acetate, and the product isolated by TLC (acetonitrile:-water, 96:4). The identity and deuterium content of the product were confirmed by mass spectral analysis as previously described [1].

2.3. Measurement of anandamide levels in cell culture and in blood

Anandamide and NAGly levels were measured in plasma extracts by HPLC–MS/MS with d_8-anandamide added as an internal standard. Reverse-phase HPLC was performed on a 100× 1 mm i.d. column packed with ODS Hypersil (3 μm, 120 Å pore size; Keystone Scientific, Inc.). A Rheos 2000 micro HPLC pumping system was used to pump the mobile phase (90% methanol, 10% of 0.05% aqueous ammonium acetate buffer, pH 5.7) at 50 μL/min. The outlet from the column was connected directly to the electrospray ion source of a Finnigan LCQ quadrupole ion trap mass spectrometry system. Positive ion electrospray ionization was used with the source at 4500 V, the capillary at 200°, and the nitrogen sheath gas at a relative setting of 60. NAGly eluted at 1.4 min and was detected by MS^2 of its MH^+ ion (*m/z* 362.2) with an isolation window of 2.5 Th and relative collision energy (CID) of 29%. Full product ion spectra were collected from *m/z* 95–370, and peak areas from ion plots of *m/z* 287.2 were used for quantitation. Anandamide and d_8-anandamide were similarly detected using their MH^+ ions (*m/z* 348.2 and 356.2) as precursors with isolation windows of 2.5 Th and CID at 30%. Full product ion spectra were collected from *m/z* 200–370, and peak areas from ion plots of *m/z* 286.2 and 292.2 were used for anandamide and d_8-anandamide, respectively. Concentrations of NAGly and anandamide were calculated from their peak area ratios to the internal standard with reference to an external calibration curve.

2.4. Statistical analysis

Values are the means ± SD for 4 replicates unless indicated otherwise. A one-factor ANOVA evaluation was used followed by a Fisher PLSD post hoc test to compare the different sets of experimental data for statistical significance. The sets were considered to be significantly different at $P < 0.05$.

3. Results and discussion

3.1. Elevation of anandamide levels in RAW cells

In this study, evidence was sought to determine whether NAGly would cause a rise in anandamide levels in a

physiologically relevant system, namely, an intact cell model such as the cultured macrophage RAW 264.7 cell line. In addition, experiments were also done in RAW cells to rule out the possibility that NAGly might serve as a metabolic precursor for anandamide, an effect that would result in an increase in anandamide concentration [1]. Treatment of RAW cells with 10 μM NAGly caused a 50% elevation of basal concentrations of anandamide, as determined by mass spectral analysis (Fig. 2A). To exclude the possibility that NAGly was a precursor for the increased anandamide, the experiment was repeated except that only deuterium-labeled NAGly (d_8-NAGly) was used in the treatment. The label was contained entirely in the arachidonyl portion of the molecule (see Section 2). Fig. 2B shows the data obtained, indicating that virtually all of the 50% increase (~3 ng/mL) in anandamide consists of unlabeled material. Only an insignificant increase (<0.5 ng/mL) in d_8-anandamide was found to occur when the cells were treated with d_8-NAGly.

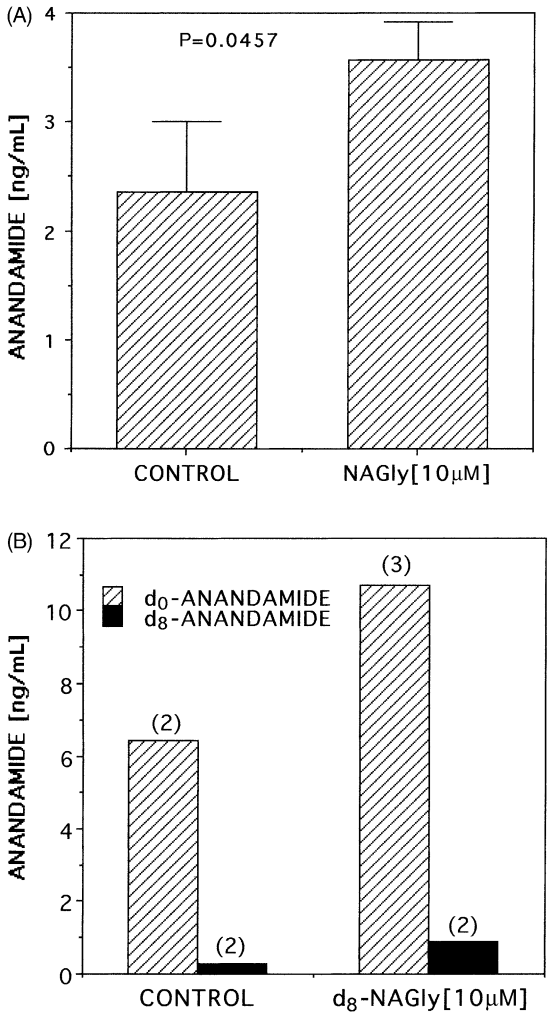

Fig. 2. Effect of NAGly on anandamide levels in intact cultured RAW 264.7 cells. (A) Treatment with unlabeled NAGly. Cells were grown as described previously [14] and treated for 60 min as described in Section 2. Values shown for anandamide were obtained by mass spectrometric analysis and are means ± SD (N = 4). Data were obtained using non-deuterated NAGly [10 μM]. (B) Comparison with deuterium-labeled NAGly. Cells were grown and treated exactly as in (A) except that treatment was done only with deuterated NAGly [10 μM]. Mass spectrometric analyses were performed for both deuterated and non-deuterated anandamide as described in Section 2. The value shown for d_8-anandamide in the control was essentially due to background noise. Numbers in parentheses are the number of samples analyzed.

The increase in anandamide levels in the RAW cells following NAGly exposure (Fig. 2) is in agreement with its previously reported inhibitory action on FAAH activity in cell membranes [1]. However, evidence was not obtained that would conclusively establish a role for FAAH in the effects observed in the present study. FAAH catalyzes the hydrolysis of fatty acid amide bonds such as that found in anandamide and, therefore, is considered to be a physiological control point in maintaining cellular anandamide concentrations [10,13]. In any case, the data presented here, using an intact cell culture model, add support to the hypothesis that NAGly may function as a pharmacological regulator of *in vivo* anandamide levels. The possibility that the increased anandamide might be due to free arachidonic acid resulting from the hydrolysis of NAGly is precluded by the lack of labeled anandamide found when deuterated NAGly was used as the agonist (Fig. 2B).

3.2. *In vivo* NAGly-induced elevation of circulating levels of anandamide in rats

An extension of the cell culture experiments to an *in vivo* model was carried out to determine whether the oral administration of NAGly to rats would result in an increase in blood levels of anandamide. *In vivo* pharmacological support for the hypothesis was obtained by demonstrating increased circulating blood levels of anandamide in rats following the oral administration of NAGly (Fig. 3). A preliminary experiment showed that the administration of 10 mg/kg of NAGly to rats resulted in a rapid rise in blood levels of NAGly (60–80 pmol/mL), suggesting that a significant fraction of an orally administered dose would reach potential tissue target sites. Using a protocol similar to that reported for a study on the anandamide transport inhibitor AM404 [15], experiments using 10 mg/kg of NAGly p.o. were performed in the study reported here. Mass spectroscopic analyses showed a more than 9-fold rise in blood levels of anandamide 45 min after NAGly administration when compared with vehicle-treated animals (Fig. 3). The result shown is representative of that obtained in two separate experiments. At this time it is not known how tissue levels of endogenous NAGly are regulated; however, when these factors are understood better, it may be possible to determine whether it has a role as a physiological regulator of anandamide levels. In any event, the findings reported here suggest that NAGly is a useful template molecule for the design of pharmacological agents to control anandamide levels.

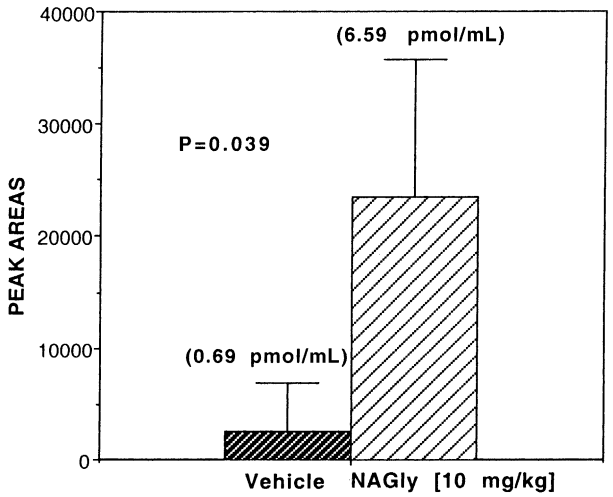

Fig. 3. Effect of orally administered NAGly on blood levels of anandamide in rats. Male Sprague–Dawley rats (150 g, N = 3) were given NAGly (10 mg/kg) p.o. and compared with a control group (N = 3) given 50 μL of safflower oil. Blood samples were drawn 45 min later, and their anandamide concentrations determined by mass spectroscopic analysis as described in Section 2. The values shown are means of the peak areas ± SD of 2–3 determinations per sample. The equivalent concentrations in pmol/mL of blood are shown in brackets. An ANOVA comparison gave a *P* value of 0.039.

3.3. Conclusions

To summarize, data have been presented that further characterize the actions of NAGly, a member of a family of naturally occurring long chain acyl amino acid conjugates. The results obtained here with both *in vitro* and *in vivo* pharmacological models demonstrate that NAGly is an effective agent for increasing tissue anandamide concentrations. Furthermore, studies with analogs of NAGly could lead to novel analgesic and anti-inflammatory agents whose actions may involve modulation of endocannabinoid tissue concentrations. The mechanism underlying our observations might arise from one or more of several possible actions of NAGly that could affect anandamide concentrations either *in vitro* or *in vivo*. For example, in addition to reducing the activity of FAAH, NAGly (a) could inhibit anandamide transport, (b) could act on the vanilloid receptor to modulate calcium ion concentrations, or (c) might down-regulate the expression level of FAAH. Di Marzo *et al*. [16] recently reported that the endogenous substance palmitoylethanolamide at 1–10 μM reduces anandamide hydrolysis by the reduction of FAAH expression. Based on our previous report [1], the first possibility seems the most likely for NAGly; however, further experimentation will be needed to conclusively establish this point.

Acknowledgments

The assistance of James E. Evans, Director, Mass Spectrometry Facility, University of Massachusetts Medical School, is gratefully acknowledged. This publication was made possible by Grants DA09439 (S.H.B.), DA09017 (S.H.B.), AR38501 (R.B.Z.), K02DA00375 (J.M.W.), NS33247 (J.M.W.), and DA13012 (J.M.W.) from The National Institute on Drug Abuse. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institute on Drug Abuse. Financial support from Sepracor Inc. (R.B.Z. and S.H.B.) is acknowledged.

References

[1] Huang SM, Bisogno T, Petros TJ, Chang SY, Zavitsanos PA, Zipkin RE, Sivakumar R, Coop A, Maeda DY, De Petrocellis L, Burstein S, Di Marzo V, Walker JM. Identification of a new class of molecules, the arachidonyl amino acids, and characterization of one member that inhibits pain. J Biol Chem 2001;276:42639–44.

[2] Burstein S, Pearson W, Rooney T, Yagen B, Zipkin R, Zurier A. Studies with analogs of anandamide and indomethacin. In: Symposium on the cannabinoids, July 1997. Burlington, VT: International Cannabinoid Research Society, 1997. p. 31.

[3] Pertwee RG. Cannabinoid receptors and pain. Prog Neurobiol 2001;63:569–611.

[4] Walker JM, Huang SM, Strangman NM, Tsou K, Sanudo-Pena MC. Pain modulation by release of the endogenous cannabinoid anandamide. Proc Natl Acad Sci USA 1999;96:12198–203.

[5] Pertwee RG. The ring test: a quantitative method for assessing the 'cataleptic' effect of cannabis in mice. Br J Pharmacol 1972;46:753–63.

[6] Sheskin T, Hanus L, Slager J, Vogel Z, Mechoulam R. Structural requirements for binding of anandamide-type compounds to the brain cannabinoid receptor. J Med Chem 1997;40:659–67.

[7] Burstein SH. The cannabinoid acids: nonpsychoactive derivatives with therapeutic potential. Pharmacol Ther 1999;82:87–96.

[8] Huang SM, Bisogno T, Petros TJ, Chang SY, Zavitsanos PA, Zipkin RE, Sivakumar R, Coop A, Maeda DY, DePetrocellis L, Burstein SH, DiMarzo V, Walker JM. The isolation and identification of *N*-arachidonylglycine: a novel endogenous neuroregulator. In: Symposium on the cannabinoids, July 2001. Burlington, VT: International Cannabinoid Research Society, 2001. p. 78.

[9] Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR, Lichtman AH. Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. Proc Natl Acad Sci USA 2001;98:9371–6.

[10] Di Marzo V. Biosynthesis and inactivation of endocannabinoids: relevance to their proposed role as neuromodulators. Life Sci 1999;65:645–55.

[11] Di Marzo V, Bisogno T, De Petrocellis L, Melck D, Orlando P, Wagner JA, Kunos G. Biosynthesis and inactivation of the endocannabinoid 2-arachidonoylglycerol in circulating and tumoral macrophages. Eur J Biochem 1999;264:258–67.

[12] Burstein SH, Rossetti RG, Yagen B, Zurier RB. Oxidative metabolism of anandamide. Prostaglandins Other Lipid Mediat 2000;61:29–41.

[13] Di Marzo V, Deutsch DG. Biochemistry of the endogenous ligands of cannabinoid receptors. Neurobiol Dis 1998;5(6 Pt B):386–404.

[14] Pestonjamasp VK, Burstein SH. Anandamide synthesis is induced by arachidonate mobilizing agonists in cells of the immune system. Biochim Biophys Acta 1998;1394:249–60.

[15] Giuffrida A, Rodriguez de Fonseca F, Nava F, Loubet-Lescoulie P, Piomelli D. Elevated circulating levels of anandamide after administration of the transport inhibitor, AM404. Eur J Pharmacol 2000;408:161–8.

[16] Di Marzo V, Melck D, Orlando P, Bisogno T, Zagoory O, Bifulco M, Vogel Z, De Petrocellis L. Palmitoylethanolamide inhibits the expression of fatty acid amide hydrolase and enhances the anti-proliferative effect of anandamide in human breast cancer cells. Biochem J 2001;358:249–55.