Biosynthesis and turnover of anandamide and other *N*-acylethanolamines in peritoneal macrophages

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Received 5 August 1999; received in revised form 2 September 1999

Abstract Polyunsaturated *N*-acylethanolamines (NAEs), including anandamide (20:4*n*−6 NAE), elicit a variety of biological effects through cannabinoid receptors, whereas saturated and monounsaturated NAEs are inactive. Arachidonic acid mobilization induced by treatment of intact mouse peritoneal macrophages with Ca^{2+} ionophore A23187 had no effect on the production of NAE or its precursor *N*-acylphosphatidylethanolamine (*N*-acyl PE). Addition of exogenous ethanolamine resulted in enhanced NAE synthesis by its *N*-acylation with endogenous fatty acids, but this pathway was not selective for arachidonic acid. Incorporation of ^{18}O from $H_2{}^{18}O$-containing media into the amide carbonyls of both NAE and *N*-acyl PE demonstrated a rapid, constitutive turnover of both lipids.

Key words: *N*-Acylethanolamine; *N*-Acyl PE; Anandamide; Macrophage; *N*-Acyl turnover

1. Introduction

Long-chain *N*-acylethanolamines (NAEs) occur in a variety of plant and animal tissues and in certain microorganisms, usually as trace constituents (reviewed in [1]). In mammalian tissues, they accumulate post mortem [2] and under pathological conditions involving degenerative membrane changes [1]. It appears that under such conditions a loss of calcium homeostasis results in the activation of a transacylase that catalyzes the direct transfer of *O*-acyl groups from the *sn*-1 position of glycerophospholipids to the amino group of ethanolamine phospholipids [3]. The resulting *N*-acylphosphatidylethanolamines (*N*-acyl PE) are then cleaved by a phosphodiesterase of the phospholipase D type to phosphatidic acid and NAE [4].

Because the *sn*-1 position of glycerophospholipids is occupied primarily by saturated and monounsaturated fatty acids, the 'transacylation-phosphodiesterase pathway' produces primarily saturated and monounsaturated NAEs [1]. However, polyunsaturated NAEs, including anandamide (20:4*n*−6 NAE), also occur in small proportions among mammalian NAE and they were shown to bind to and activate cannabinoid receptors, whereas saturated NAE was inactive [5,6]. In order for anandamide to function as a physiological agonist, the mechanisms of its biosynthesis and degradation must be subject to regulation. At this time, little is known about such metabolic regulation although it is now well established that anandamide can be synthesized through transacylation via *N*-arachidonoyl PE [7], provided that arachidonic acid is present at the *sn*-1 position of a donor phospholipid [8] (reviewed in [9]). Hence, the generation of phospholipid molecular species having arachidonic acid at the *sn*-1 position may be an important first step in anandamide synthesis.

It has long been known that 1,2-diarachidonoyl glycerophospholipids occur in small amounts in mammalian cells and we have recently shown that they can be generated through the de novo biosynthetic pathway from exogenous [10] or endogenously generated arachidonic acid [11]. We have observed enhanced generation of 1,2-diarachidonoyl PC in mouse peritoneal macrophages treated with the Ca^{2+} ionophore A23187 and have proposed that this enhancement could result in the selective generation of anandamide [10,11]. Because mouse peritoneal macrophages contain both *N*-acyl PE and NAE, including anandamide [12], and because NAE signalling may play a role in the immune system [9,13], we have investigated possible mechanisms of selective anandamide synthesis in these cells in the presence and absence of A23187 and in the presence and absence of exogenous ethanolamine.

2. Materials and methods

2.1. Materials

Male mice (ICR) were obtained from Clear Japan (Tokyo, Japan). Thioglycolate was from Difco; Eagle's minimum essential medium (MEM) was from Nissui Pharmaceutical Company (Tokyo, Japan). Water enriched in ^{18}O (98 atom %) and [1,1,2,2-$^2H_4$]ethanolamine were purchased from Isotec (Miamisburg, OH, USA). Unlabeled ethanolamine and calcium ionophore A23187 were from Sigma (St. Louis, MO, USA). [1,2-^{14}C]Ethanolamine (specific activity, 2.5 mCi/mmol) was from NEN Life Science Products (USA). L-Glutamine and all other chemicals used in the incubation experiments were analytical grade from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Preparation and incubation of macrophages

Peritoneal exudate cells, consisting primarily of macrophages (>80%), were prepared from mice (8–10 weeks old) that had been injected i.p. with 2 ml of sterile 3% aqueous thioglycolate (Difco) as described previously [14]. The mice were killed by decapitation 4 days after the injection and the cells were harvested by lavage of the peritoneal cavity with Dulbecco's phosphate buffered saline [PBS(−)], pH 7.2, containing 10 units of heparin/ml and no Ca^{2+} and Mg^{2+}. Cells were passed through stainless steel mesh (200 mesh), pooled and checked by trypan blue exclusion (>90% viability). They were

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Abbreviations: NAE, *N*-acylethanolamine; *N*-acyl PE, *N*-acylphosphatidylethanolamine; TLC, thin-layer chromatography; GC/MS, gas-chromatography/mass spectrometry

0014-5793/99/$20.00
PII: S0014-5793(99)01226-0

washed twice with [PBS(−)] and resuspended in MEM containing kanamycin, supplemented with L-glutamine, at densities of 1.0×10^8 cells per ml. Aliquots of the cell suspension were extracted [15] or incubated in the presence of 5 μM A23187 (in ethanol) and/or different concentrations of ethanolamine (in saline). Control incubations contained either ethanol (final concentration, 0.25%) or saline. Incubations were carried out in duplicate using 0.2 ml of cell suspension added to 0.8 ml of MEM containing up to 10 μl of 100 mM ethanolamine. In one experiment, aliquots of the cell suspension were taken for determination of the diameter of macrophages, untreated or treated with 1.4 mM ethanolamine and/or 5 μM A23187 for 30 min at 37°C. In the radioisotope experiments, the cells were incubated with increasing concentrations of unlabeled ethanolamine in the presence of 1 μCi of [^{14}C]ethanolamine for 30 min at 37°C. In experiments involving $H_2{}^{18}O$, doubly-concentrated MEM (0.4 ml) was mixed with $H_2{}^{18}O$ (0.4 ml) and preincubated for 5 min in the presence or absence of d_4 ethanolamine (1 mM) before the addition of the cell suspension. In most cases, incubations were carried out in duplicate at 37°C. They were terminated by adding 1 ml of ice-cold PBS(−) and placing the cell suspension in an ice bath for 5 min. Cells and media were either extracted together according to Folch et al. [15] as described [12] or separated by centrifugation at 1200 rpm for 5 min at 4°C. This was done for the experiments involving $H_2{}^{18}O$ in which the supernatant was removed and lipid extracts of only the cells were prepared. Lipid extracts were dissolved in 1 ml of toluene/ethanol (4:1, v/v) and shipped to the Hormel Institute for lipid fractionation and analysis.

2.3. Lipid fractionation and analysis

To each sample was added a mixture of deuterated NAEs (0.1 μg each of 16:0, 17:0, 18:0, 18:1*n*−9, 18:2*n*−6 and 20:4*n*−6) and 1 nmol of *N*-heptadecanoyl-1,2-dioleoylphosphatidylethanolamine. Small aliquots were taken for assay of lipid phosphorus [16]. NAE and *N*-acyl PE were separated by thin-layer chromatography (TLC), derivatized with *tert*-butyldimethylchlorosilane/imidazole reagent (Alltech) and analyzed by gas-chromatography/mass spectrometry (GC/MS) as previously described [12].

2.4. Determination of cell volume

Aliquots of cell suspensions were diluted with the same buffer, applied into a glass chamber and photographed under a microscope. The pictures were enlarged and printed at a final magnification of 233 times. In each photograph, the diameter of more than 200 cells was measured using a caliper. The average cell volume was calculated from the average cell diameter.

2.5. Assay of ^{18}O incorporation

No d_4 NAE was added to the extracts of cells that had been incubated with d_4 ethanolamine and $H_2{}^{18}O$. NAE and *N*-acyl PE were isolated and derivatized as usual for GC-MS. Selected ion monitoring allowed the measurement of 16:0, 18:1*n*−7, and 18:0 NAEs containing: (a) no stable isotopes; (b) one ^{18}O atom; (c) four deuterium atoms from d_4 ethanolamine; and (d) four deuterium atoms plus one ^{18}O atom as described previously [17]. Because of their incomplete separation and the resultant interference in the *m*+2 ions, isotope enrichment in 18:2*n*−6 and 18:1*n*−9 NAEs could not be measured. Anandamide (20:4*n*−6 NAE) was not present in sufficient quantity to provide accurate measurements. The ratios of ions containing ^{18}O to ions containing ^{16}O were calculated for each NAE (Table 2).

3. Results

3.1. Effects of ionophore treatment on the levels and compositions of NAE and N-acyl PE

Based on the results of our previous work with these cells [11,12] and data obtained with other macrophages [18–23], we expected that ionophore-mediated Ca^{2+} uptake into mouse peritoneal macrophages would not only result in enhanced *N*-acyl PE synthesis by the Ca^{2+}-dependent transacylation pathway but could also lead to the generation of increased proportions of 20:4*n*−6 in both *N*-acyl PE and NAE. The latter assumption was based on the observed Ca^{2+}-mediated mobilization of arachidonic acid and its re-incorporation via 1,2-diarachidonoyl phospholipids [11].

However, incubation of cells for 15 min in MEM with or without 5 μM A23187 had no measurable effects on the levels or compositions of either NAE or *N*-acyl PE (data not shown). Because cells and media were extracted together, any NAEs released from the cells would have been analyzed together with intracellular NAE or *N*-acyl PE. As reported

Table 1
NAE and *N*-acyl PE in mouse peritoneal macrophages incubated with or without ethanolamine and with or without A23187

	pmol/μmol phospholipid P							
NAE	16:0	18:0	18:1*n*−9	18:1*n*−7	18:2*n*−6	20:4*n*−6	Total	20:4 as % of total
No incubation	7.6	5.7	1.3	1.4	0.7	0.2	**16.9**	1.18
No ethanolamine or A23187, 30 min	9.6	7.4	2.0	2.1	1.6	0.4	**23.1**	1.73
No ethanolamine, 5 μM A23187	10.2	5.8	1.5	1.8	1.2	0.6	**21.1**	2.84
0.1 mM ethanolamine, no A23187	13.2	9.3	3.2	2.5	2.6	0.7	**31.5**	2.22
0.1 mM ethanolamine, 5 μM A23187	13.3	10.7	4.2	2.4	3.8	0.9	**35.3**	2.55
0.5 mM ethanolamine, no A23187	24.0	17.2	8.7	3.6	8.0	1.8	**63.3**	2.84
0.5 mM ethanolamine, 5 μM A23187	29.9	21.4	11.6	3.9	11.4	2.0	**80.2**	2.49
N-acyl PE								
No incubation	78.8	40.9	26.3	7.7	29.7	3.4	**186.8**	1.82
No ethanolamine or A23187	51.8	45.3	21.1	6.2	21.1	3.2	**148.7**	2.15
No ethanolamine, 5 μM A23187	66.0	52.0	24.8	6.1	25.4	3.8	**178.1**	2.13
0.1 mM ethanolamine, no A23187	46.6	41.3	18.4	6.6	21.5	4.2	**138.6**	3.03
0.1 mM ethanolamine, 5 μM A23187	75.5	60.5	33.4	9.5	35.9	5.1	**219.9**	2.32
0.5 mM ethanolamine, no A23187	47.5	41.0	20.1	6.7	23.1	3.9	**142.3**	2.74
0.5 mM ethanolamine, 5 μM A23187	57.7	47.8	25.1	7.9	28.0	4.3	**170.8**	2.51

Cells were incubated in MEM for 15 min with or without ethanolamine in the indicated concentrations. Then 5 μM A23187 dissolved in 2.5 μl of ethanol was added and incubation was continued for 15 min. Cells and medium were extracted together. NAE and *N*-acyl PE were analyzed by GC-MS as described in Section 2.

previously for these cells [12], *N*-palmitoyl moieties amounted to about one-half of the total, whereas *N*-arachidonoyl moieties ranged from 0.75 to 1.69% in NAE and from 2.96 to 3.26% in *N*-acyl PE.

We conclude that Ca^{2+}-activated fatty acid turnover and arachidonic acid liberation, under conditions previously shown to cause enhanced production of 1,2-diarachidonoyl PC [11], does not affect the levels or compositions of either NAE or *N*-acyl PE in mouse peritoneal macrophages.

3.2. Effects of exogenous ethanolamine on the levels and compositions of NAE and N-acyl PE

When mouse peritoneal macrophages were incubated with increasing concentrations of ethanolamine, NAE levels were increased but *N*-acyl PE levels and their *N*-acyl compositions were unaffected (Table 1). Among NAE, the relative proportion of anandamide (20:4*n*−6 NAE) remained about the same. Also, NAE levels in the presence of ethanolamine plus A23187 were higher than in the presence of ethanolamine alone. We interpret these data to indicate that uptake of ethanolamine into the cells was followed by its direct *N*-acylation with intracellular fatty acids. It is possible that intracellular fatty acid levels were somewhat higher in the presence of the Ca^{2+} ionophore, resulting in higher levels of NAE through *N*-acylation, but there appeared to be no selectivity for anandamide synthesis under the experimental conditions used. It is logical to assume that any excess of free arachidonic acid, generated by ionophore treatment, was metabolized by these cells through reacylation, oxidation and/or release into the medium without becoming available for enhanced anandamide synthesis through the *N*-acylation of ethanolamine.

3.3. Effects of exogenous ethanolamine on cell diameter and [^{14}C]ethanolamine incorporation

We expected that exogenous ethanolamine was freely taken up into the cells but it was of interest to establish intracellular ethanolamine concentrations and to determine possible effects of the relatively high ethanolamine levels on cell shape and viability. We therefore repeated the incubation with ^{14}C-labeled ethanolamine in the presence and absence of A23187, separated the cells from the media and determined average cell diameter under the microscope, cell viability by trypan blue exclusion, and internal ethanolamine concentration by radioassay of cells and comparison with the cell volume. In all instances, neither ethanolamine nor A23187 treatment had measurable effects on cell size (diameter 13.83 ± 1.85 μm to 14.65 ± 2.06 μm), viability (91.3% to 97.6%) and the intracellular ethanolamine concentration was approximately the same or slightly higher than that of the incubation media (data not shown).

3.4. Mechanisms of N-acylation

In order to determine whether the exogenously added ethanolamine was directly *N*-acylated with endogenous fatty acids, we added fully deuterated ethanolamine and incubated the cells in $H_2{}^{18}O$-containing media. Any presence of ^{18}O in the d_4-ethanolamine-containing NAE would indicate direct *N*-acylation. However, we expected that only d_4 ethanolamine-labeled NAEs would contain ^{18}O in their amide-linked fatty acids, whereas NAEs generated from PE via *N*-acyl PE would not. Indeed, previous experiments with particulate preparations of dog heart [24] or rat testes [17] had shown that the transacylase-catalyzed acyl transfer resulting in the *N*-acylation of PE occurred without ester hydrolysis and did not cause incorporation of ^{18}O into the amide-linked fatty acids of either *N*-acyl PE [24] or the NAE [17] derived from it.

However, when intact macrophages were incubated in 40% $H_2{}^{18}O$ in the presence of d_4 ethanolamine, we found ^{18}O incorporated into NAE derived from both exogenous (deuterated) and endogenous (unlabeled) ethanolamine. Because it was likely that the precursor of the endogenous (non-deuterated) NAE was *N*-acyl PE, produced from PE by *N*-acylation, we determined the ^{18}O content of the amide-linked fatty acids of *N*-acyl PE.

As shown in Table 2, both *N*-acyl PE and NAE of macrophages incubated for 30 min in 40% $H_2{}^{18}O$ contained substantial amounts of ^{18}O in their amide-linked fatty acids. Because of the potential importance of this observation, we repeated the experiment in the presence of either d_4 ethanolamine or unlabeled ethanolamine and obtained essentially the same results (Table 2).

Incorporation of ^{18}O into NAE and *N*-acyl PE indicates that constitutive *N*-acylation of PE and its hydrolysis to NAE involves unesterified fatty acids produced through ester hydrolysis. The similar high levels of ^{18}O in *N*-acyl PE and NAE might be due to the presence of a small pool of acyl donor phospholipids exhibiting a rapid rate of turnover thus providing ^{18}O-labeled 1-*O*-acyl groups for transacylation to the amino groups of PE [1]. Alternatively, the transacylation occurring in intact cells could occur by a mechanism that results in oxygen exchange with the media, or *N*-acylation of PE in these macrophages could proceed by a novel mech-

Table 2
Atom % excess of ^{18}O in amide-linked fatty acids of macrophage NAE and *N*-acyl PE after 30 min of incubation in media containing 40% $H_2{}^{18}O$ plus 1 mM d_4 ethanolamine or unlabeled ethanolamine[a]

NAE						*N*-acyl PE		
N-acyl ethanolamine			*N*-acyl (d_4) ethanolamine			*N*-acyl ethanolamine		
16:0	18:0	18:1*n*-7	16:0	18:0	18:1*n*-7	16:0	18:0	18:1*n*-7
first experiment (d_4 ethanolamine)								
4.0, 3.3	8.7, 4.6	12.1, 7.8	16.0, 19.5	15.2, 17.5	3.7, 2.9	11.4, 15.5	21.7, 24.8	6.9, 10.0
second experiment (d_4 ethanolamine)								
10.1, 12.1	13.5, 18.9	6.9, 5.1	17.0, 17.9	14.0, 16.1	4.5, 3.7	14.3, 15.9	23.1, 25.5	8.3, 7.9
second experiment (ethanolamine)								
15.4, 17.6	20.6, 25.7	7.7, 7.9	–	–	–	15.2, 19.9	21.5, 26.1	7.5, 6.8

Data represent the results of two incubations analyzed separately.
[a]Cells were incubated, lipids were extracted, NAE and *N*-acyl PE were isolated, derivatized and analyzed as described in Section 2. 18:1*n*−9, 18:2*n*−6 and 20:4*n*−6 NAE could not be analyzed as explained in Section 2.

anism involving free fatty acid or acyl CoA. Our current data do not allow us to distinguish between these possibilities and further work will be required.

4. Discussion

Our present results show (1) that treatment of mouse peritoneal macrophages with Ca^{2+} and ionophore A23187 has no measurable effect on the levels and compositions of *N*-acyl PE and NAE, (2) that exogenous ethanolamine can be directly *N*-acylated by endogenous fatty acids and (3) that substantial amounts of ^{18}O appear in the amide-linked fatty acids of both *N*-acyl PE and NAE when cells are incubated in $H_2{}^{18}O$-containing media, suggesting very rapid turnover of the amide-linked fatty acids in both lipid classes.

Previous work by others had shown that ionomycin treatment of neuronal cells [7,20,21], mouse J774 [19,20] and RAW264.7 [23] macrophages or leukocytes [21], prelabeled with [^{14}C]ethanolamine or various radioactive fatty acids, results in the generation of labeled NAEs, including anandamide. A labeled polar lipid was also generated and was identified as *N*-acyl PE by chemical analysis. However, when the distribution of radioactivity among the free NAEs and those derived from *N*-acyl PE were compared, they were found to be substantially different [21]. Interestingly, anandamide synthesis was found to be induced in prelabeled RAW264.7 macrophages by conditions that caused arachidonic acid mobilization including ionophore treatment [23]. However, if and how the liberated arachidonic acid participates in anandamide synthesis remains unclear.

Because ionophore treatment of mouse peritoneal macrophages results not only in the liberation of arachidonic acid but also in its re-uptake into phospholipids in part through de novo synthesis via 1,2-diarachidonoyl PC and PE [11], we have suggested that these molecular species could serve as acyl donors for the selective generation of anandamide through the *N*-acylation-phosphodiesterase pathway [10,11]. Although this view has been adopted by others [9], no direct evidence has been provided and our present data argue against it. In fact, Ca^{2+} entry into macrophages through ionophore treatment had no measurable effect on the levels and compositions of either *N*-acyl PE or NAE.

In contrast, the addition of ethanolamine resulted in a substantial increase of macrophage NAE levels through its direct acylation with endogenous fatty acids. This is suggested by the data of Table 1 and conclusively proven by the d_4 ethanolamine experiment summarized in Table 2. We have previously demonstrated this reaction in rat testes membranes [17], but this is the first evidence that such *N*-acylation of ethanolamine can occur in intact cells. It is therefore possible that the simultaneous generation of both arachidonic acid and ethanolamine through phospholipase A_2 and phospholipase D activity as suggested earlier [25,26] may, in fact, be a biologically relevant pathway for the selective synthesis of anandamide. So far no 'NAE synthase' has been identified, but there exists ample evidence that the NAE amidohydrolase can act in reverse, catalyzing the synthesis of various NAEs from ethanolamine and fatty acid in vitro [27–30]. Hence, it is possible that this enzyme could utilize locally generated ethanolamine and arachidonic acid for the selective synthesis of anandamide.

Perhaps most importantly, our present results show the very rapid incorporation of ^{18}O from $H_2{}^{18}O$-containing media into the carbonyls of the amide-linked fatty acids of both *N*-acyl PE and NAE. We have previously determined *O*-acyl turnover of mouse peritoneal macrophage phospholipids by the $H_2{}^{18}O$ technique and have observed nearly linear ^{18}O incorporation for 1 h, reaching levels of 1–4 atom % excess [14]. Considering the concentration of $H_2{}^{18}O$ (40%) and the loss of one oxygen during fatty acid acylation, we estimated *O*-acyl turnover in these cells to reach up to 20% per h. In the present situation such estimates cannot be made because atom % excess of ^{18}O in some cases (e.g. the amide of 18:0 NAE and *N*-acyl PE) already exceeded 25% after 30 min and the mechanism of ^{18}O incorporation remains uncertain (see above). The nearly identical distribution of ^{18}O atom % excess in 16:0, 18:0 and 18:1*n*-7 NAE (from endogenous ethanolamine) and the corresponding *N*-acyl PE (Table 2, last line) strongly suggests that NAE synthesis in these cells proceeds via *N*-acyl PE, as seen in all other mammalian systems studied so far [1,9]. The extremely rapid turnover of macrophage *N*-acyl PE suggests a dynamic equilibrium between the *N*-acylation of PE and its degradation by phospholipase D activity [4] to NAE, which in turn must be degraded by amidohydrolase [27–30]. It can be assumed that *N*-arachidonoyl groups participate in this sequence even though their amounts were too small to be measured in the present ^{18}O assay. We propose that agonists other than elevated Ca^{2+} levels are needed to elicit enhanced NAE synthesis through this reaction sequence. In view of the fact that the cannabinoid receptor-inactive saturated and monounsaturated NAEs usually constitute more than 95% of all NAEs produced in most mammalian cells, it is possible that the main purpose of the *N*-acylation-phosphodiesterase pathway is the production of saturated/monounsaturated NAEs whose functional significance remains to be determined.

Acknowledgements: This work was supported in part by NIH Grant GM45741 and by The Hormel Foundation.

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