Age dependent accumulation of *N*-acyl-ethanolamine phospholipids in ischemic rat brain: a ^{31}P NMR and enzyme activity study

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Abstract ***N*-acyl-ethanolamine phospholipids (NAPE) can be formed as a stress response during neuronal injury, and they are precursors for *N*-acyl-ethanolamines (NAE), some of which are endocannabinoids. The levels of NAPE accumulated during post-decapitative ischemia (6 h at 37°C) were studied in rat brains of various age (1, 6, 12, 19, 30, and ~70 days) by the use of ^{31}P NMR spectroscopy of lipid extracts. This ability to accumulate NAPE was compared with the activity of *N*-acyltransferase and of NAPE-hydrolyzing phospholipase D (NAPE-PLD) in brain microsomes. These two enzymes are involved in the formation and degradation of NAPE, respectively. The results showed that *1*) the ability to accumulate NAPE during post-decapitative ischemia is especially high in the youngest rats and is markedly reduced in older brains [in 1-day-old rat brains NAPE accumulated to 1.5% of total phospholipids, while in 30-day-old rat brains NAPE accumulation could not be detected (detection limit 0.09 %)]; and *2*) this age pattern of accumulation can be explained by a combination of the decreased activity of *N*-acyltransferase and the increased activity of NAPE-PLD during development. These results point out that it would be advantageous to investigate a potential cytoprotective role of NAPE in the brains of very young rats.**—Moesgaard, B., G. Petersen, J. W. Jaroszewski, and H. S. Hansen. **Age dependent accumulation of *N*-acyl-ethanolamine phospholipids in ischemic rat brain: a ^{31}P NMR and enzyme activity study.** ***J. Lipid Res.*** 41: **985–990.**

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The formation of *N*-acyl-ethanolamines (NAE) and their precursors *N*-acyl-ethanolamine phospholipids (NAPE) is considerably enhanced in certain injured cells where the level of intracellular Ca^{2+} is elevated (1, 2). This may be the case under pathophysiological conditions like hypoxia and ischemia and in neurodegenerative diseases. Under the conditions of hypoxia/ischemia the endogenous excitotoxic amino acids glutamate (Glu) and aspartate accumulate extracellularly in the CNS. A part of the neuronal degeneration that is taking place is thought to be caused by the action of Glu at the NMDA subtype of Glu receptors (3, 4). Studies of primary cultures of mouse cortical neurons showed a Glu-induced formation of NAPE and NAE mediated via activation of the NMDA-receptor, and that the ability to form these two lipids is dependent on the maturity of the cell cultures (5–7). Earlier, in vivo studies have shown that the vulnerability of the immature rat brain to NMDA-induced neurotoxicity increased during the early neonatal period (days 2–4), reached a peak at day 6, and then diminished progressively with age (4).

Although the biological functions of NAPE and NAE still remain to be elucidated, the formation of these lipids, including the endocannabinoid *N*-arachidonoyl-ethanolamine (also called anandamide) (8, 9), is suggested to serve a cytoprotective role (1). Formation of NAPE is catalyzed by a Ca^{2+}-activated *N*-acyltransferase that utilizes ethanolamine phospholipids as acceptor substrates for the transfer of an *sn*-1 acyl group from a donor phospholipid, e.g., phosphatidylcholine (2). NAPE can be hydrolyzed by a NAPE-specific phospholipase D (NAPE-PLD) and thereby generate NAE and phosphatidic acid (2, 10). NAPE-PLD does not seem to discriminate between different *N*-acyl groups (10, 11). Some of the NAE species, e.g., anandamide, can function as endogenous ligands for cannabinoid receptors (12, 13). Both *N*-acyltransferase and NAPE-PLD are membrane associated and have a high activity in rodent brains (2, 11, 14). Activity of rat brain *N*-acyltransferase has

Abbreviations: BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; CL, cardiolipin; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; Glu, glutamate; lyso-PE, ethanolamine lysophospholipids; NAE, *N*-acyl-ethanolamines; NAPE, *N*-acyl-ethanolamine phospholipids; $NAPE_{DIACYL}$, 1,2-diacyl-*sn*-glycero-3-phospho(*N*-acyl)ethanolamine; $NAPE_{PLAS}$, 1-(1′-alkenyl)-2-acyl-*sn*-glycero-3-phospho(*N*-acyl)ethanolamine; NAPE-PLD, NAPE-hydrolyzing phospholipase D; PA, phosphatidic acid; PC_{DIACYL}, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PE_{AA}, 1-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine; PE_{DIACYL}, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; PE_{PLAS}, 1-(1′-alkenyl)-2-acyl-*sn*-glycero-3-phosphoethanolamine; PI, inositol phospholipids; PMSF, phenylmethylsulfonyl fluoride; PS, serine phospholipids; SPH, sphingomyelin.

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previously been measured at four time points during development (days 2, 10, 15, and 30) and that of NAPE-PLD at only two time points (days 10 and 30) (15). These limited data indicated that the activity of *N*-acyltransferase initially was low, then increased, and later decreased again with age, whereas the activity of NAPE-PLD was higher at day 30 than at day 10. The post-decapitative ischemia-induced accumulation of NAPE was found to be more pronounced in developing rat brain (10-days-old) than in adult brain (15). We have previously shown that post-decapitative accumulation of NAPE can be quantified by ^{31}P NMR (16). Our initial studies showed high accumulation of NAPE in the brains of very young rats (1-day-old), which is in contrast to the low activity of *N*-acyltransferase reported by Natarajan, Schmid, and Schmid (15). Therefore, the present work was performed in order to obtain more detailed information about the developmental course in rat brains with respect to *1*) post-decapitative accumulation of NAPE, *2*) the activity of *N*-acyl-transferase, and *3*) the activity of NAPE-PLD.

MATERIALS AND METHODS

Animals and materials

Six groups of Sprague-Dawley male rats of the age 1, 6, 12, 19, 30, or ~70 days were obtained from our own stable. All commercial chemicals were used as received. CH_3OH, $CHCl_3$, $CaCl_2$, KOH, dry dimethyl sulfoxide (DMSO), and TLC plates (Silica Gel 60) were obtained from Merck (Darmstadt, Germany). 1,2-Didecanoyl-*sn*-glycero-3-phosphocholine, Na-EDTA (disodium salt), K-EDTA (dipotassium salt), sucrose, Tris-HCl, 1,3-bis[tris (hydroxymethyl)methylamino]propane (BTP), dithiothreitol (DTT), 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine, palmitic acid, phenylmethylsulfonyl fluoride (PMSF), 1,1′-carbonyldiimidazole, and 4-dimethylaminopyridine were purchased from Sigma Chemical Co. (St. Louis, MO). Triton X-100 was obtained from Boehringer Mannheim Gmbh (Mannheim, Germany). $CDCl_3$ (99.8%) was from the Cambridge Isotope Laboratories (Andover, MA). [1-^{14}C]palmitic acid (55 Ci/mol) and 1,2-di[1′-^{14}C]decanoyl-*sn*-glycero-3-phosphocholine (67 Ci/mol) were obtained from Amersham Pharmacia Biotech (Amersham, UK). Ammonia solution was obtained from Aldrich (Steinheim, Germany). Aqueous solutions of K-EDTA were adjusted to pH 6.0 with aqueous KOH. H-buffer was prepared as 25 mm Tris-HCl, 0.25 m sucrose, and 2 mm Na-EDTA adjusted to pH 7.3. BTP-buffer was prepared as 60 mm BTP adjusted to pH 8.0 or 8.25 with HCl.

Lipid extraction

For the study of post-decapitative ischemia-induced accumulation of NAPE, rats were decapitated and the whole heads were wrapped in foil and immediately incubated at 37°C for 6 h and thereafter extracted. For controls, brains were excised within 2 min after decapitation of the rats, placed on ice, and within 15 min frozen and kept at −80°C until used for extraction. The lipids were extracted from excised brains by a modified (16) procedure of Folch, Lees, and Sloane Stanley (17). Two samples of lipid extract were made from each group of rat brains. Each sample contained an extract from one brain except for samples from 1- and 6-days-old brains where four or two brains, respectively, were pooled before homogenization and extraction. The extracts were added 0.2 ml of 0.1 m aqueous K-EDTA per ml of $CHCl_3$–CH_3OH 2:1 (v/v) and mixed thoroughly before being transferred to long glass tubes and left at 4°C overnight in order to separate the phases. The lower organic phases were isolated and evaporated to dryness under a stream of nitrogen and the total amounts of lipids were determined. The lipids were redissolved in $CHCl_3$–CH_3OH 2:1 (v/v) and kept at −20°C until used for preparation of ^{31}P NMR samples as described below.

^{31}P NMR analysis

NMR samples were made from duplicates of brain lipid extracts from 1-, 6-, 12-, 19-, 30-, and ~70-days-old rats. Appropriate volumes of the lipid extract, containing 19–23 mg of lipids, were transferred to small vials with airtight screwcaps. The solvent was evaporated to dryness under a stream of nitrogen and the lipids were redissolved in 700 μl of a mixture of $CDCl_3$, CH_3OH, and 0.1 m aqueous K-EDTA 100.0:29.9:5.2 (v/v/v). The NMR samples were stored tightly closed at −20°C until used. The NMR spectrometer used was a Bruker AMX 400 WB system operating at 161.98 MHz for ^{31}P. Analytical samples were placed in standard (5 mm) NMR sample tubes spun at 20 Hz during the accumulation of data. Proton broad-band decoupling was effected by the Walz-16 decoupling sequence. The ^{2}H NMR signal of $CDCl_3$ was used for field-frequency locking. Homogeneity of the magnetic field was adjusted for the ^{1}H signal of $CHCl_3$ to a typical line width of 0.6 Hz (measured at half height). Probe temperature was stabilized at 25°C. Each NMR spectrum was the sum of 4096 free induction decays with a total accumulation time of 2 h. The transients were typically collected as 16,384 complex data points during an acquisition period of 0.82 s. The spectral width was 10,000 Hz. The spin-flip angle used was 60° (8.5 μs), and the relaxation delay was 1.0 s. The chemical shifts were standardized relative to the chemical shift of the naturally occurring phospholipid 1,2-diacyl-*sn*-glycero-3-phosphocholine (PC_{DIACYL}) set to −0.84 ppm (18). Data processing was performed on Lorentz-Gauss transformed spectra with a line broadening of −2.0 Hz and a Gaussian broadening of 0.5 Hz. The area of the signals was determined using the standard Bruker software. The conditions used for the determination of the ^{31}P NMR spectra were optimized for the maximal signal-to-noise ratio for a given experimental time. According to earlier experiments (16) the spectra obtained under these conditions are satisfactory to follow changes in the content of NAPE subclasses in the rat brain lipid extracts. The area of the ^{31}P NMR signals of 1,2-diacyl-*sn*-glycero-3-phospho(*N*-acyl)ethanolamine ($NAPE_{DIACYL}$) plus 1-(1′-alkenyl)-2-acyl-*sn*-glycero-3-phospho(*N*-acyl)ethanolamine ($NAPE_{PLAS}$) was determined as the percentage of the total area of the phospholipid signals.

Isolation of rat brain microsomes

For the study of the activity of *N*-acyltransferase and of NAPE-PLD in brain microsomes, rats were decapitated and the brains excised within 2 min and washed with an ice-cold Krebs-glucose solution. The brains were frozen within 15 min and kept at −80°C until used for subcellular fractionation. Two samples of microsomes were made from each group of rat brains. Each sample consisted of microsomes from one brain except for samples from 1- and 6-days-old brains where four or two brains, respectively, were pooled before homogenization and isolation of microsomes. Brains were homogenized in H-buffer (1:6 w/v) with Ultra Turrax T25. Afterwards, H-buffer was added up to a total ratio of 1:10 w/v. The homogenates were spun at 1000 *g* for 10 min at 4°C to remove tissue debris, and then at 109,000 *g* for 1 h. The pellets containing the microsomes were carefully suspended in an appropriate volume of BTP-buffer (pH 8.25) corresponding to a final protein concentration of 20–30 mg per ml. Protein determinations were performed by the method of Bradford (19)

modified for membrane samples (boiling with NaOH). Suspensions of microsomes were frozen in small aliquots and kept at −80°C until used for enzyme assays.

Determination of *N*-acyltransferase activity

Enzyme preparations (100 μg protein) were incubated (in triplicates) with 1.2 nmol of 1,2-di[1′-^{14}C]decanoyl-PC (1.5 x 10^5 dpm/nmol) for 0, 10, 20, and 30 min at 37°C in a total volume of 250 μl of BTP-buffer (pH 8.25) containing 3 mm DTT, 5 mm $CaCl_2$, and 0.01 % Triton X-100. The reaction was terminated by addition of 1.5 ml of $CHCl_3$–CH_3OH 2:1 (v/v) and the organic phase separated. Re-extraction was performed by addition of 1 ml of $CHCl_3$. Lipids were separated by TLC with $CHCl_3$–CH_3OH–aqueous NH_3 80:20:2 (v/v/v) as the mobile phase. The distribution of radioactivity in the lipid spots was located and quantified using a PhosphoImager scanner (STORM, Molecular Dynamics, Sunnyvale, CA). The activity of *N*-acyltransferase was expressed as the amount (in pmol) of *N*-[1-^{14}C]decanoyl species of NAPE plus lyso-NAPE formed per min per mg protein during the first 20 min of the incubation during which the formation was linear with time. Each microsomal fraction was assayed twice and the activity was expressed as the mean value.

Determination of NAPE-PLD activity

The substrate 1,2-dilauroyl-*sn*-glycero-3-phospho(*N*-[1′-^{14}C] palmitoyl)ethanolamine (NAPE) was prepared essentially as described by Schmid et al. (20) using 50 μCi of [1-^{14}C]palmitic acid (0.9 μmol) diluted with unlabeled palmitic acid (21.3 μmol) to a specific activity of 5.0×10^3 dpm/nmol, and reacted with 1,1′-carbonyldiimidazole for 4 h. After the addition of 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine and 4-dimethylaminopyridine, the reaction was continued for 20 h in $CHCl_3$ at room temperature. Enzyme preparations (200 μg of protein) were incubated with 2.1 nmol of the substrate NAPE in a total volume of 200 μl BTP buffer (pH 8.0) containing 2 mm DTT, 0.04 % Triton X-100, 10 mm PMSF, and 5 μl DMSO. Incubations were carried out in triplicates at 37°C for 0, 15, 30, 60, 120, and 180 min. The reaction was terminated by adding 1.5 ml of ice-cold $CHCl_3$–CH_3OH 2:1 (v/v) and the organic phase separated. Re-extraction was performed by the addition of 1 ml of $CHCl_3$. Lipids were separated by TLC and the distribution of radioactivity determined as above. The activity of NAPE-PLD was expressed as the amount (in pmol) of *N*-[1-^{14}C]palmitoyl-ethanolamine (NAE) formed per min per mg protein during the first 120 min of the incubation during which the formation was linear with time. Each microsomal fraction was assayed twice and the activity was expressed as the mean value.

RESULTS

Accumulation of NAPE during post-decapitative ischemia

Examples of ^{31}P NMR spectra of brain lipid extracts from 6- and 30-days-old rats exposed to 6 h of post-decapitative ischemia at 37°C are shown in **Fig. 1**. The assignment of the signals of the two NAPE subclasses, $NAPE_{PLAS}$ and $NAPE_{DIACYL}$, and of the other phospholipids [ethanolamine lysophospholipids (lyso-PE), phosphatidic acid (PA), cardiolipin (CL), 1-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (PE_{AA}), 1-(1′-alkenyl)-2-acyl-*sn*-glycero-3-phosphoethanolamine (PE_{PLAS}), 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (PE_{DIACYL}), serine phospholipids (PS), sphingomyelin (SPH), and inositol phospholipids (PI)] is as previously described (16). The spectra demonstrate that after the 6 h period of post-decapitative ischemia there was a substantial accumulation of $NAPE_{PLAS}$ and $NAPE_{DIACYL}$ in the 6-days-old rat brains, whereas the accumulation in the 30-days-old rat brains did not reach the limit of ^{31}P NMR detection (0.09% of total phospholipid signal) (16) in these experiments. **Figure 2** shows the percentage of the NAPE species determined relative to the total phospholipid content, as a function of age. Control spectra of brain lipids extracted from freshly decapitated rat heads of all ages demonstrated that the content of NAPE subclasses was below the detection limit and was thus set to zero in Fig. 2. The content of NAPE during post-decapitative ischemia was reduced only slightly in 6-days-old (1.4%) compared to 1-day-old rats (1.5 %), whereas a nearly linear decrease by a factor of 5 was observed as the age increased from 6 to 19 days, where the NAPE content was 0.3% of total phospholipids. In 30- and ~70-days-old ischemic rat brains the content of the NAPE subclasses was below the detection limit.

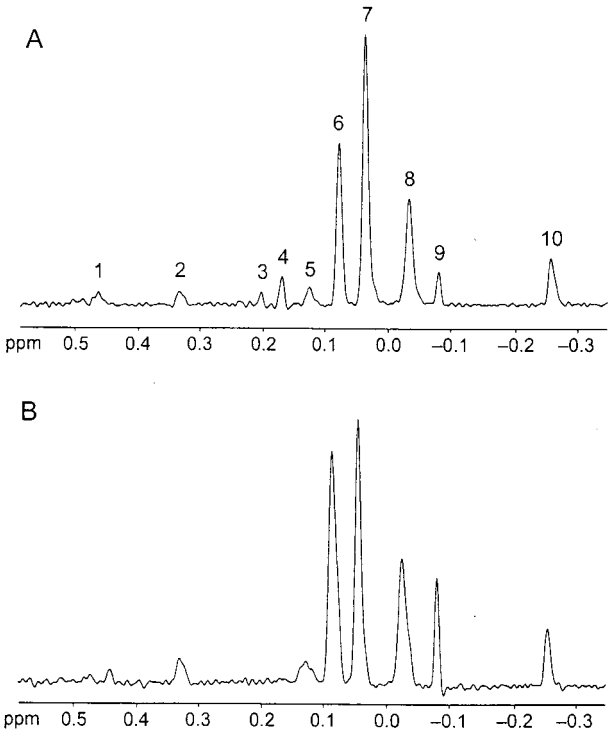

Fig. 1. ^{31}P NMR spectra (161.98 MHz) of lipids extracted from A: 6- or B: 30-days-old rat brains after exposure to post-decapitative ischemia for 6 h at 37°C. The lipid extracts were prewashed with K-EDTA, the solvents evaporated, and the lipids redissolved in $CDCl_3$–CH_3OH–0.1 m aqueous K-EDTA 100.0:29.9:5.2 (v/v/v). The NMR spectra were recorded at 25°C (4096 transients). The chemical shifts were standardized relative to the signal of PC_{DIACYL} set at −0.84 ppm. The assignments of the signals are: 1, lyso-PE-subclasses and PA (the region 0.43–0.52 ppm); 2, CL; 3, $NAPE_{PLAS}$; 4, $NAPE_{DIACYL}$; 5, PE_{AA}; 6, PE_{PLAS}; 7, PE_{DIACYL}; 8, PS; 9, SPH; 10, PI.

Activity of *N*-acyltransferase

The activity of *N*-acyltransferase was determined as the formation of *N*-[1-^{14}C]decanoyl species of NAPE plus lyso-NAPE (in pmol) per min per mg protein (**Fig. 3**, insert). Only minor amounts of *N*-[1-^{1}4C]decanoyl-ethanolamine

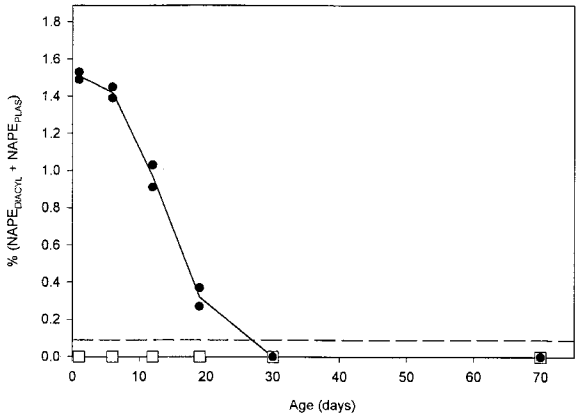

Fig. 2. Accumulation of NAPE in rat brains during 6 h of postdecapitative ischemia as measured by ^{31}P NMR spectroscopy of lipid extracts. Rats of the age 1, 6, 12, 19, 30, and ~70 days were decapitated, and the heads were incubated at 37°C for 0 h (= controls) (□) or 6 h (●) before the brains were excised, homogenized, and extracted. The areas of the NMR signals of NAPE$_{DIACYL}$ plus NAPE$_{PLAS}$ were determined as the percentage of the total area of the phospholipid signals. The amounts of NAPE species were too low to be quantified and were set to zero in ischemic brains of 30- and ~70-days-old rats and in all control rat brains. The limit of detection of 0.09% is represented by the dashed line. Each point represents data from one brain except for points for 1- and 6-days-old rats where four and two brains, respectively, were pooled before homogenization and extraction. n = 2 for both controls and ischemic brains of each age.

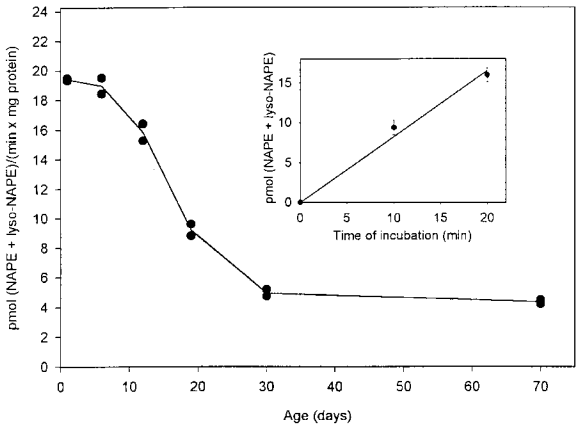

Fig. 3. Activity of *N*-acyl-transferase in brain microsomes isolated from 1-, 6-, 12-, 19-, 30-, and ~70-days-old rats, measured as pmol of *N*-[^{14}C]decanoyl species of NAPE plus lyso-NAPE (both the diacyl and the plasmalogen species) formed per min per mg protein from 1,2-di[1′-^{14}C]decanoyl-PC and endogenous ethanolamine phospholipids, and determined as shown in the inset. Each point represents data from one brain except for points for 1- and 6-days-old rats where four and two brains, respectively, were pooled before homogenization and extraction. Insert: An example of the assay of *N*-acyltransferase activity in rat brain microsomes (19-days-old rat). 100 μg of microsomal protein was incubated in triplicates for 0, 10, 20, and 30 min at 37°C, with 1.2 nmol of 1,2-di[1′-^{14}C]decanoyl-PC (1.5×10^5 dpm/nmol) in a total volume of 250 μl of BTP-buffer (pH 8.25) containing 3 mm DTT, 5 mm $CaCl_2$, and 0.01% Triton X-100. Calculation of the enzyme activity was performed on data collected during the first 20 min of incubation.

(NAE) (below 10% of NAPE plus lyso-NAPE) were observed to accumulate during the whole period of incubation (30 min) under the actual assay conditions, and since this accumulation was not linear with time, it was not included in the determination of *N*-acyltransferase activity. To ensure that the observed lack of accumulation of NAE was not caused by its active, amidase-catalyzed degradation to [1-^{14}C]decanoic acid, a test of the stability of NAPE during 30 min of incubation under the assay conditions was performed. *N*-[1-^{14}C]decanoyl-PE (NAPE) was isolated from a macroscale incubation of an enzyme preparation similar to the *N*-acyltransferase assay. Amounts of *N*-[1-^{14}C]decanoyl-PE (NAPE) similar to the maximum amount formed in the above assay were incubated with enzyme preparations isolated from either 6-, 19-, or 30-days-old rat brains. The incubations were performed under normal assay conditions except for PC which was added as 1,2-didecanoyl-PC instead of 1,2-di[1′-^{14}C] decanoyl-PC. In this experiment, the amount of NAPE plus lyso-NAPE (in dpm) was constant during the 30 min of incubation (data not shown), indicating that the activity of NAPE-PLD under these assay conditions was negligible. Furthermore, this excludes a possible interference by phospholipase A_1/A_2 during the incubations.

The activity of *N*-acyltransferase determined in the enzyme preparations isolated from the brains of rats from different groups of age is illustrated in Fig. 3. As shown in the figure, the activity of *N*-acyltransferase in brain microsomes was rather similar in 1- and 6-days-old rats (19.4 and 19.0 pmol per min per mg protein), whereas there was a nearly linear 4-fold decrease of the activity with age up to 30 days (4.9 pmol per min per mg protein).

Activity of NAPE-PLD

The activity of NAPE-PLD was determined as the amount of *N*-[1-^{14}C]palmitoyl-ethanolamine (NAE) (in pmol) formed per min per mg protein in the presence of PMSF that inhibits NAE degradation (21, 22) (**Fig. 4**, insert). The activity of NAPE-PLD as a function of age is shown in Fig. 4. A minor increase of the activity was seen when comparing microsomes isolated from 1- and 6-days-old rat brains (1.3 and 1.7 pmol per min per mg protein), after which a 9-fold increase in the activity was observed with age from 6 to 30 days (15.5 pmol per min per mg protein). When comparing brain microsomes isolated from 30- and ~70-days-old rats (19.7 pmol per min per mg protein) only a smaller increase in NAPE-PLD activity was observed.

DISCUSSION

The formation of NAPE and NAE (including anandamide) under pathophysiological conditions is suggested to serve a neuroprotective role (1), e.g., NAPE may be membrane stabilizing (23, 24) and the different NAE species may be cytoprotective via non-receptor-mediated actions, as well as via activation of cannabinoid receptors (8, 9, 25–28). The neuronal degeneration that takes place

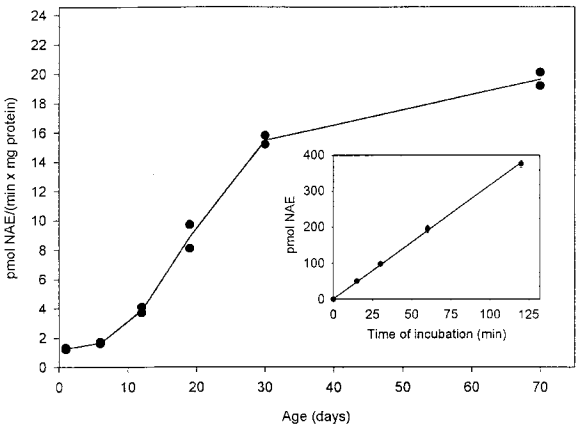

Fig. 4. Activity of NAPE-PLD in brain microsomes isolated from 1-, 6-, 12-, 19-, 30-, and ~70-days-old rats, measured as pmol of *N*-[1-^{14}C]palmitoyl-ethanolamine (NAE) formed per min per mg protein from 1,2-dilauroyl-*sn*-glycero-3-phospho(*N*-[1′-^{14}C]palmitoyl)ethanolamine (NAPE), and determined as shown in the inset. Each point represents data from one brain except for points for 1- and 6-days-old rats where four and two brains, respectively, were pooled before homogenization and extraction. Inset: An example of the assay of NAPE-PLD activity in rat brain microsomes (30-days-old rat). 200 μg of microsomal protein was incubated in triplicates for 0, 15, 30, 60, 120, and 180 min at 37°C, with 2.1 nmol of 1,2-dilauroyl-*sn*-glycero-3-phospho(*N*-[1′-^{14}C]palmitoyl)ethanolamine (NAPE) (5.0×10^3 dpm/nmol) in a total volume of 200 μl of BTP-buffer (pH 8.0) containing 2 mm DDT, 0.04% Triton X-100, 10 mM PMSF, and 5 μl DMSO. Calculation of the enzyme activity was performed on data collected during the first 120 min of incubation.

under the condition of hypoxia/ischemia is thought to be caused partially by the action of Glu at the NMDA subtype of Glu-receptors (3, 4). In vitro studies of mouse cortical neurons showed that the Glu-induced formation of NAPE and NAE was dependent on the maturity of the cell cultures (5). The present data demonstrate a high ability to accumulate NAPE in 1- and 6-days-old rat brains (Fig. 2) under post-decapitative ischemia. This ability decreased nearly linearly from the age of 6 days up to the age of 19 days, where the accumulation was reduced by a factor of 5. The NAPE-accumulating ability was closely paralleled by the activity of *N*-acyltransferase, the enzyme catalyzing the formation of NAPE, in rat brain microsomes (Fig. 3). Our results with the very young rats (1 day) are in contrast with those of Natarajan et al., who found a low activity of *N*-acyltransferase in a brain homogenate from 2-days-old rats (15). We have at present no explanation for this discrepancy but a high level of the formation of NAPE was observed in the present work both with microsomal *N*-acyltransferase activity and directly by ^{31}P NMR measurements. The small differences in the assay conditions used, e.g., Ca^{2+} concentration and pH, are estimated to be of minor importance due to the fact that constant assay conditions were used for all age-related experiments in the previous (15) and the present study.

This finding is of importance in relation to our hypothesis that NAPE/NAE formation may serve a cytoprotective role in relation to neuronal injury (1). The high activity of *N*-acyltransferase in the very young rats suggests that it would be advantageous to investigate a putative neuroprotective role of NAPE in the brains of very young animals, e.g., in models of head trauma in developing rat brain (29). In contrast, the activity of NAPE-PLD, the enzyme catalyzing the degradation of NAPE to NAE, in rat brain microsomes showed a relatively low and similar level in 1- and 6-days-old rats, while a nearly linear 9-fold increase in activity was seen in older rats up to the age of 30 days. Our more elaborate measurements of NAPE-PLD thus extend the observations of Natarajan et al. (15). The decreasing ability to accumulate NAPE under (post-decapitative) ischemia with age seems to be caused both by a decreasing activity of *N*-acyltransferase and by an increasing activity of NAPE-PLD with age. Thus, NAPE can be formed in adult rat brain but it can be expected from the present study that it may be used for the generation of NAE. Schmid et al. (30) have shown that different species of NAE accumulated post mortem in the adult brains of cow, sheep, and pigs.

In summary, the present study suggests that NAPE may play a role in the young developing rat brain, possibly as a cytoprotective phospholipid, whereas the ability to form NAE is much more pronounced in the adult rat brain, suggesting that NAE may have a role in the mature brain, possibly as a group of neuroprotective lipid messengers.

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