Accumulation of *N*-acyl-ethanolamine phospholipids in rat brains during post-decapitative ischemia: a ^{31}P NMR study

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Abstract **Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) spectroscopy has been used to study accumulation of *N*-acyl-ethanolamine phospholipids in rat brains during post-decapitative ischemia. Lipids were extracted from rat brain homogenates and the extracts were thoroughly washed with aq. potassium ethylenediaminetetraacetic acid (EDTA). The lower organic phases were isolated and evaporated to dryness under a stream of nitrogen and the lipids were redissolved in $CDCl_3$–CH_3OH–H_2O 100.0:29.9:5.2 (v/v/v) for NMR analysis. Increasing the period of post-decapitative ischemia resulted in an accumulation of two signals in the NMR spectra at 0.18 and 0.22 ppm (relative to the chemical shift of 1,2-diacyl-*sn*-glycero-3-phosphocholine (PC_{DIACYL}) at −0.84 ppm). These signals were identified as originating from 1,2-diacyl-*sn*-glycero-3-phospho-(*N*-acyl)-ethanolamine ($NAPE_{DIACYL}$) and 1-(1′-alkenyl)-2-acyl-*sn*-glycero-3-phospho-(*N*-acyl)-ethanolamine ($NAPE_{PLAS}$), respectively, by spiking with authentic materials. Additionally, the identification was verified by thin-layer chromatography, which also showed the accumulation of *N*-acyl-ethanolamine phospholipids. The use of K-EDTA instead of the commonly used Cs-EDTA in the preparation of the NMR samples allowed the separation of the chemical shifts of *N*-acyl-ethanolamine phospholipids from those of the ethanolamine phospholipids. Moreover, the chemical shift of cardiolipin was moved from 0.15 ppm observed with Cs-EDTA to about 0.31 ppm with K-EDTA. The present study demonstrates that it is possible to detect and quantify post-decapitative accumulation of NAPE subclasses ($NAPE_{DIACYL}$ and $NAPE_{PLAS}$) in rat brains by the use of ^{31}P NMR spectroscopy.—**Moesgaard, B., J. W. Jaroszewski, and H. S. Hansen. **Accumulation of *N*-acyl-ethanolamine phospholipids in rat brains during post-decapitative ischemia: a ^{31}P NMR study. *J. Lipid Res.* 1999.** 40: **515–521.**

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The accumulation of *N*-acyl-ethanolamine phospholipids (NAPE) under the condition of post-decapitative ischemia has earlier been reported in rat brains using a chromatographic method (1). This rare lipid is of biological interest because it is the precursor for *N*-acyl-ethanolamines, some of which are ligands for the cannabinoid receptors (2), as well as because this class of phospholipids by itself may have membrane stabilizing properties and their accumulation may indicate neuronal injury (2, 3). Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) spectroscopy is a convenient method for the determination of phospholipids (4), and the chemical shifts of many phospholipids extracted from biological tissues, including cardiolipin (CL), 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (PE_{DIACYL}), 1-(1′-alkenyl)-2-acyl-*sn*-glycero-3-phosphoethanolamine (PE_{PLAS}), ethanolamine lysophospholipids (LPE), 1-2-diacyl-*sn*-glycero-3-phosphocholine (PC_{DIACYL}), 1-(1′-alkenyl)-2-acyl-*sn*-glycero-3-phosphocholine (PC_{PLAS}), 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (PC_{AA}), choline lysophospholipids (LPC), sphingomyelin (SPH), serine phospholipids (PS), inositol phospholipids (PI), and phosphatidic acid (PA) have been characterized (4–13). To our knowledge, NAPE has not previously been detected in biological samples by use of ^{31}P NMR. It was our objective to identify and quantify NAPE in extracts of animal tissue by the use of ^{31}P NMR.

Abbreviations: CL, cardiolipin; EDTA, ethylenediaminetetraacetic acid; FID, free induction decay; LPC, choline lysophospholipids; LPE, ethanolamine lysophospholipids; NAPE, *N*-acyl-ethanolamine phospholipids; $NAPE_{AA}$, 1-alkyl-2-acyl-*sn*-glycero-3-phospho-(*N*-acyl)-ethanolamine; $NAPE_{DIACYL}$, 1,2-diacyl-*sn*-glycero-3-phospho-(*N*-acyl)-ethanolamine; $NAPE_{PLAS}$, 1-(1′-alkenyl)-2-acyl-*sn*-glycero-3-phospho-(*N*-acyl)-ethanolamine; $NAPE_{TRIOLEOYL}$, 1,2-dioleoyl-*sn*-glycero-3-phospho-(*N*-oleoyl)-ethanolamine; NMR, nuclear magnetic resonance; PA, phosphatidic acid; PC, choline phospholipids; PC_{AA}, 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine; PC_{DIACYL}, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PC_{PLAS}, 1-(1′-alkenyl)-2-acyl-*sn*-glycero-3-phosphocholine; PE, ethanolamine phospholipids; 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; PS, serine phospholipids; SPH, sphingomyelin; T_1, longitudinal NMR relaxation time; T_2*, apparent transverse NMR relaxation time; TLC, thin-layer chromatography.

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METHODS

Animals

For the study of post-decapitative ischemia-induced accumulation of NAPE, Sprague-Dawley rats (10 days old, mixed sexes) were purchased from Møllergaard Breedings, Lille Skensved, Denmark. The rats were decapitated and the whole heads were wrapped in foil and immediately incubated at 37°C for different periods of time. Control rats were decapitated and the brains were excised within 1 min and kept in $CHCl_3$–CH_3OH 2:1 (v/v) on ice, until homogenized and extracted (within 15 min) as described below.

For the identification of the ^{31}P NMR signals of NAPE-subclasses and cardiolipin, Sprague-Dawley rats (8 weeks old, mixed sexes) were obtained from our own animal stable. The rats were decapitated and the brains were excised within 1 min and kept in $CHCl_3$–CH_3OH 2:1 (v/v) on ice, until homogenized and extracted (within 15 min) as described below.

Materials

All commercial chemicals were used as received without further purification. CH_3OH, $CHCl_3$, $CuSO_4$, KOH (all analytical grade reagents) and thin-layer chromatography (TLC) plates (Silica Gel 60) were obtained from Merck (Darmstadt, Germany). EDTA (free acid), K-EDTA (dipotassium salt), CsOH, and CL (bovine heart) were purchased from Sigma Chemical Co. (St. Louis, MO). Aqueous solutions of Cs-EDTA were obtained by titration of EDTA (free acid) with CsOH to pH 6.0. Aqueous solutions of K-EDTA were adjusted to pH 6.0 with aqueous KOH. $CDCl_3$ (99.8%) was from the Cambridge Isotope Laboratories (Andover, MA). Ammonia solution and orthophosphoric acid were obtained from Aldrich (Steinheim, Germany) and Riedel-de Haën AG (Seelze-Hannover, Germany), respectively. The mixture of *N*-decanoyl species of the NAPE subclasses [1,2-diacyl-*sn*-glycero-3-phospho-(*N*-acyl)-ethanolamine ($NAPE_{DIACYL}$), 1-(1′-alkenyl)-2-acyl-*sn*-glycero-3-phospho-(*N*-acyl)-ethanolamine ($NAPE_{PLAS}$), and 1-alkyl-2-acyl-*sn*-glycero-3-phospho-(*N*-acyl)-ethanolamine ($NAPE_{AA}$)] were synthesized from bovine brain ethanolamine phospholipids (PE) [containing a mixture of PE_{DIACYL}, PE_{PLAS}, and PE_{AA} (14)] and decanoyl chloride using a modification of the procedures described by Epps et al. (15) and Sugiura et al. (16). PE and decanoyl chloride were purchased from Sigma Chemical Co. (St. Louis, MO). 1,2-Dioleoyl-*sn*-glycero-3-phospho-(*N*-oleoyl)-ethanolamine ($NAPE_{TRIOLEOYL}$) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL).

Lipid extraction

The brains were excised from the rat heads and lipids were extracted by a modified (6, 10) procedure of Folch, Lees, and Sloane Stanley (17). The brains (weighing ~700 mg) were homogenized on ice in 20 times amount (v/w) of $CHCl_3$–CH_3OH 2:1 (v/v) with Ultra Turrax T25 (Janke & Kunkel IKA-Labortechnik, Staufen, Germany). The homogenates were placed on ice for 2 h to settle and filtered through a funnel packed with glass wool. The extracts, 0.2 ml of either 0.1 m aq. K-EDTA or 0.1 m aq. Cs-EDTA (pH 6.0) per ml of $CHCl_3$–CH_3OH 2:1 (v/v), were added 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 amount of lipids was determined. The lipids were redissolved in $CHCl_3$–CH_3OH 2:1 (v/v) and kept at −20°C until used. Henceforward the lipids will be referred to as either the K- or the Cs-salts of the lipids depending on whether the lipid extracts have been prewashed with K-EDTA or Cs-EDTA.

^{31}P NMR analysis

Appropriate volumes of the lipid extracts, containing 20–25 mg of lipids (K- or Cs-salts), were transferred to small vials with airtight screwcaps. The solvents were evaporated under a stream of nitrogen and the lipids were redissolved in 700 μl of a mixture of $CDCl_3$, CH_3OH, and either H_2O, 0.2 m aq. K-EDTA (pH 6.0), or 0.2 m aq. Cs-EDTA (pH 6.0) 100.0:29.9:5.2 (v/v/v). The NMR samples were stored tightly closed at −20°C until used.

The standards of phospholipids (CL, $NAPE_{TRIOLEOYL}$, and a mixture of the three NAPE-subclasses) were washed with either K-EDTA or Cs-EDTA according to Branca et al. (18). Thus about 0.5 mg of CL or $NAPE_{TRIOLEOYL}$, or about 1.0 mg of the mixture of NAPE-subclasses was dissolved in 500 μl of $CHCl_3$, mixed with 200 μl of CH_3OH and 50 μl of 0.2 m aq. K- or Cs-EDTA (pH 6.0) and vortexed for about 5 min. The solutions were left at 4°C overnight in order to separate the phases. The organic phases containing the phospholipids were isolated and evaporated under a stream of nitrogen. The K- or Cs-salts of the phospholipids were redissolved in $CHCl_3$ and kept at −20°C, until mixed with rat brain lipids and used for the preparation of NMR samples as described above.

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 Waltz-16 decoupling sequence. The ^2H NMR signal of $CDCl_3$ was used for field-frequency locking. Homogeneity of the magnetic field was adjusted for the ^1H 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 (FIDs) with total accumulation time of 2 h. The FIDs were typically collected as 16384 complex data points during an acquisition period of 0.82 s. The spectral width was 10000 Hz. The spin-flip angle used was 60° (8.5 μs), and the relaxation delay was 1.0 s. Occasionally, spectra with the relaxation delay of 12.0 s were obtained. The longitudinal relaxation times (T_1) were measured using the inversion recovery method and were found to be 1.5 s and 1.3 s for the signals of PE_{DIACYL} and PE_{PLAS}, respectively. The chemical shifts were standardized relative to the chemical shift of the signal of the naturally occurring phospholipid PC_{DIACYL} set to −0.84 ppm (6). Data processing was performed on Lorentz-Gauss transformed spectra with a line broadening of −1.0 Hz and a Gaussian broadening of 0.5. The area of the signals was determined using the standard Bruker software.

Thin-layer chromatography

Two samples, each containing about 0.5 mg of lipids (K-salts) extracted from a control brain and from a brain exposed to post-decapitative ischemia for 6 h at 37°C were evaluated by TLC with $CHCl_3$–CH_3OH–aq. NH_3 80:20:2 (v/v/v) as the developing solvent. CL or $NAPE_{TRIOLEOYL}$ (10 μg samples) were used as standards. The spots were visualized by spraying with a 10% (w/w) solution of copper sulfate in 85% orthophosphoric acid and charred at 200°C.

RESULTS AND DISCUSSION

^{31}P NMR spectra of lipid extracts from rat brains exposed to post-decapitative ischemia

Examples of ^{31}P NMR spectra of rat brain lipids (K-salts) are shown in **Fig. 1**. The top (A) spectrum was obtained with the lipids from a control brain (lipids extracted immediately after the decapitation) and the bottom (B) spectrum was obtained with the lipids extracted from a rat

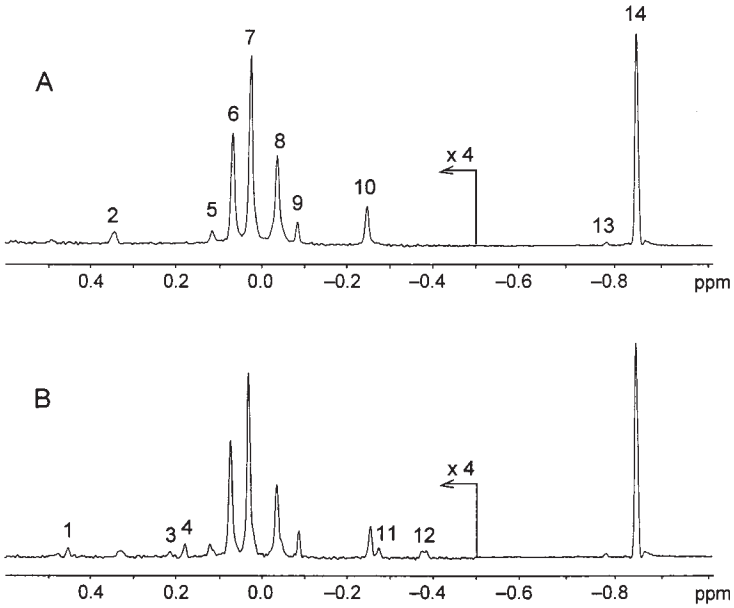

Fig. 1. ^{31}P NMR spectra (161.98 MHz) of phospholipids extracted from 10-day-old rat brains excised and homogenized either immediately after decapitation (A) or after the exposure to post-decapitative ischemia for 6 h at 37°C (B). The lipid extracts were prewashed with K-EDTA, the solvents were evaporated under a stream of nitrogen, and the lipids were redissolved in $CDCl_3$–CH_3OH–H_2O 100:29.9:5.2 (v/v/v). The NMR spectra were recorded at 25°C (4096 transients). The region from −0.5 to 0.5 ppm is plotted with four times higher vertical scale. The chemical shifts are standardized relative to the signal of PC_{DIACYL} set at −0.84 ppm. The assignments of the signals are: 1 (the region 0.43–0.52 ppm), LPE-subclasses and PA; 2, CL; 3, $NAPE_{PLAS}$; 4, $NAPE_{DIACYL}$; 5, PE_{AA}; 6, PE_{PLAS}; 7, PE_{DIACYL}; 8, PS; 9, SPH; 10, PI; 11, LPC; 12, unknown; 13, PC_{AA}; 14, PC_{DIACYL}.

brain exposed to 6 h of post-decapitative ischemia at 37°C. The spectra demonstrate that during the 6-h period of post-decapitative ischemia there was an accumulation of phospholipids giving the signals labeled 1 (0.43–0.52 ppm), 3 (0.22 ppm), 4 (0.18 ppm), 11 (−0.27 ppm), and 12 (−0.38 ppm), while the other signals remain at a rather constant level during the period of observation.

Identification of signals of NAPE-subclasses and of CL

In ^{31}P NMR spectra of lipid extracts CL is very often, if not always, reported to have resonance at 0.18 ppm relative to PC_{DIACYL} at −0.84 ppm in the $CHCl_3$–CH_3OH–aq. Cs-EDTA or K-EDTA system, and independent of whether the lipid extracts have been prewashed with KCl, K- or Cs-EDTA (4, 6–13). However, in the present work, ^{31}P NMR spectra of the K-salts of the lipids extracted from freshly excised rat brains in the $CHCl_3$–CH_3OH–H_2O system displayed no signal at 0.18 ppm, but the appearance of a signal at this chemical shift was observed during post-decapitative ischemia (Fig. 1). Spiking of the solutions with $NAPE_{TRIOLEOYL}$ or CL and redetermination of the ^{31}P NMR spectra (not shown) showed that the signal observed at 0.18 ppm originated from $NAPE_{DIACYL}$ (= $NAPE_{TRIOLEOYL}$), and that CL had resonance at about 0.34 ppm. This is in contradiction to the earlier reports stating that the chemical shift of CL is 0.18 ppm irrespective of the counterion K^+ or Cs^+ (4, 6, 12). Therefore we decided to compare ^{31}P NMR spectra of brain lipids that were prepared as K- or Cs-salts by carefully washing the lipid extracts with either K- or Cs-EDTA (pH 6.0). The results are shown in **Fig. 2**. Spectra in (A) and (D) were obtained with lipids extracted from rat brains using Cs-EDTA and K-EDTA, respectively. The remaining spectra were obtained for the brain lipids with added authentic CL [Fig. 2 (B) and (E)] or a mixture of NAPE subclasses [Fig. 2 (C) and (F)]. It is apparent that the chemical shift value of CL is about 0.15 ppm when using Cs-EDTA (B) and about 0.31 ppm when using K-EDTA [Fig. 2 (E) and other experiments (spectra not shown)]. We cannot explain the discrepancy between these findings and the earlier reported value of the chemical shift of CL of 0.18 ppm, regardless of whether the countercation is Cs^+ or K^+ (4, 6, 12). There are small differences among the chemical shift values of CL in Fig. 1 (0.34 ppm) and in Fig. 2 (0.31 ppm), which could be due to the presence of K-EDTA in the NMR solvent system. Additionaly, there is a small difference between the reported chemical shift of CL of 0.18 ppm in the Cs-EDTA system (4–13) and the value of 0.15 ppm observed in the present work (Fig. 2). This could be due to differences in the concentration of lipids in the NMR samples or differences in the solvent composition in the NMR samples.

For spiking lipid extracts with NAPE [Fig. 2 (C) and (F)], a material synthesized from decanoyl chloride and bovine brain PE was used. Bovine brain PE fraction is stated by Sigma to contain about 60% of PE_{PLAS}. The synthesis was therefore expected to produce about 40% $NAPE_{DIACYL}$ and 60% $NAPE_{PLAS}$. As seen in Fig. 2 (F), $NAPE_{DIACYL}$ and $NAPE_{PLAS}$ gave rise to two signals, at about 0.16 and 0.19 ppm, respectively. Additionally, a minor signal at about 0.22 ppm was observed upon addition of the synthetic mixture of NAPE-subclasses. This minor signal may arise from 1-alkyl-2-acyl subclass of NAPE ($NAPE_{AA}$), if the commercial bovine brain PE contained not only PE_{DIACYL} and PE_{PLAS} as reported by the supplier, but also PE_{AA}. Indeed, Malewicz and Baumann (14) estimated the composition of a commercial bovine brain PE product by ^{31}P NMR in $CDCl_3$–CH_3OD–D_2O 50:50:15 (v/v/v). Their results demonstrate that the commercial bovine brain PE consisted of 64.0 ± 1.2 mol % PE_{PLAS}, 31.8 ± 0.5 mol % PE_{DIACYL}, and 4.2 ± 0.5 mol % PE_{AA}. The relative areas of the three signals of $NAPE_{PLAS}$, $NAPE_{DIACYL}$, and $NAPE_{AA}$ observed in a spectrum (not shown) of lipids added to the synthetic mixture of NAPE subclasses was 61:34:5. This is indeed in good accordance with the results of Malewicz and Bau-

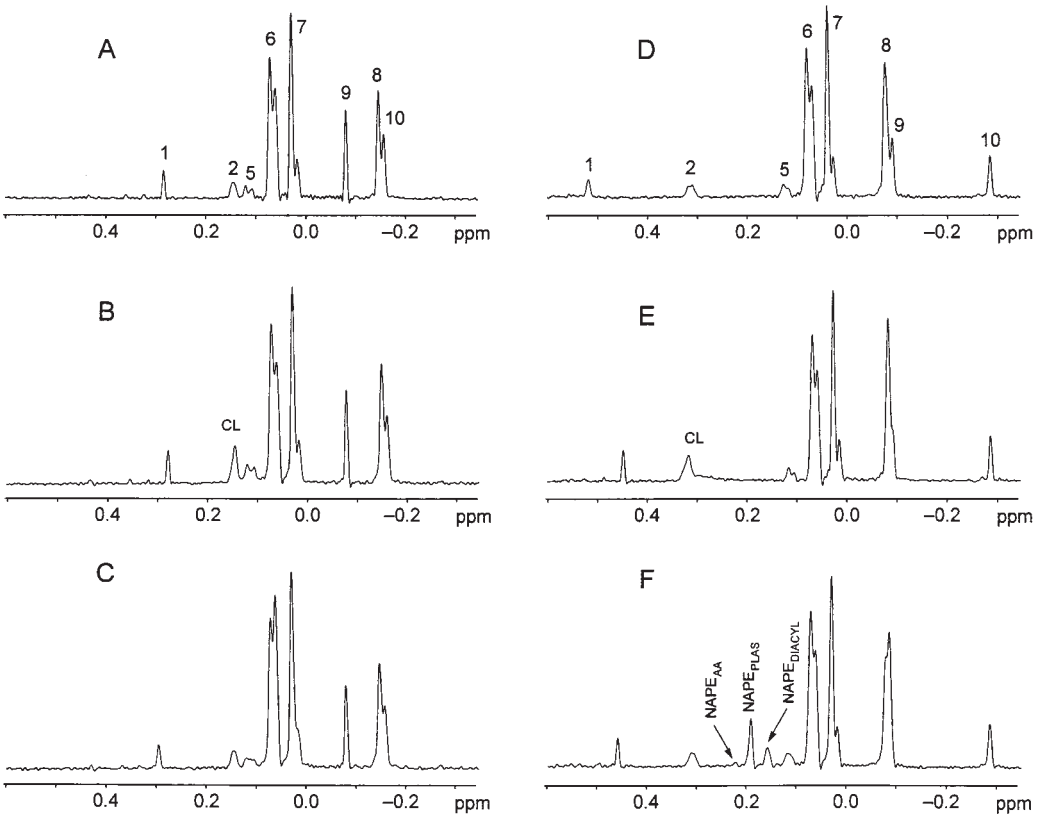

Fig. 2. ^{31}P NMR spectra (161.98 MHz) of lipids extracted from 8-week-old rat brains, spiked with standard phospholipids. Either Cs-EDTA (A–C) or K-EDTA (D–F) was used in the washing procedure of the extracts. The solvents in the lipid extracts were evaporated under a stream of nitrogen and the lipids (total 21–22 mg) were redissolved in 700 μl $CDCl_3$–CH_3OH–aq.Cs-EDTA (A–C) or aq. K-EDTA (D–F) (0.2 m, pH 6.0) 100:29.9:5.2 (v/v/v). (A) and (D): rat brain lipids. (B) and (E): rat brain lipids plus 0.5 mg CL. (C) and (F): rat brain lipids plus 1 mg of *N*-decanoyl-ethanolamine phospholipids (a mixture of the three NAPE subclasses). The spectra were recorded at 25°C (4096 transients). The chemical shifts are standardized relative to the signal of PC_{DIACYL} set at −0.84 ppm. The assignments of signals are as in Fig. 1.

mann (14). When comparing spectra (C) and (F) in Fig. 2, one will notice that NAPE is not seen as separate signals in the spectrum of the Cs-salts of the lipids. ^{31}P NMR spectra (not shown) of mixtures of Cs-salts of the phospholipids PC, PS, SPH, CL, and NAPE and of cholesterol demonstrated that the resonances of the NAPE subclasses coincide with the corresponding resonances of PE subclasses (i.e., $NAPE_{DIACYL}$ coincides with PE_{DIACYL}, etc.). This explains the apparent lack of separate signals from the different NAPE subclasses in Fig. 2 (C). However, a change in the peaks of ethanolamine phospholipids can be seen in Fig. 2 (C) as compared to (A) and (B) upon spiking with *N*-acyl-ethanolamine phospholipids.

Comparing the spectra shown in Fig. 1 and Fig. 2 one will notice minor variations in the chemical shifts and line widths of the phospholipids. These variations may, as mentioned above, be due to the use of H_2O instead of K-EDTA, minor variations in the NMR solvent compositions, and differences in the lipid concentration. It has been shown previously that the NMR solvent hydration as well as the amount of lipids affects the chemical shifts (6). However, these factors and the presence of K-EDTA in the NMR solvent system appear not to be of major importance for obtaining good quality ^{31}P NMR spectra as long as the lipid extracts have been thoroughly prewashed with K-EDTA.

Tentative assignment of the other phospholipid resonances in the ^{31}P NMR spectra of rat brain lipid extracts

We have made a tentative assignment of the remaining resonances in the ^{31}P NMR spectra (Fig. 1), based on earlier studies of phospholipids (4–13, 19) and individual lipid classes of whole rat brains (20). The signals in the region 0.43–0.52 ppm (labeled 1) may include LPE-subclasses and PA. A ^{31}P NMR spectrum (not shown) of a rat brain lipid extract spiked with PA, dissolved in $CDCl_3$–CH_3OH–H_2O 100.0:29.9:5.2 (v/v/v) showed a resonance of PA at 0.52 ppm. Signal 2 (0.34 ppm) is derived from CL and signals 3 and 4 (0.22 and 0.18 ppm) are derived from $NAPE_{PLAS}$ and $NAPE_{DIACYL}$, respectively, according to our own data. Signals 6 (0.08 ppm) and 7 (0.03 ppm) are derived from PE_{PLAS} and PE_{DIACYL}, respectively. We propose that the signal 5 (0.12 ppm) arises from PE_{AA}, because the chemical shift separation of the three signals is in accordance with the spectrum of bovine brain PE (containing PE_{AA}, PE_{PLAS}, and PE_{DIACYL}) published by Malewicz and

Baumann (14). That the signal at about 0.12 ppm corresponds to PE_{AA} has already been proposed by Metz and Dunphy (13) and Edzes et al. (19). A resonance at 0.12 ppm has earlier been shown to be derived from dihydro-SPH by Byrdwell et al. (21), but the identification was performed on lipid extracts from eye lenses and dihydro-SPH has not yet been reported to occur in brain tissue. Signals 8 (−0.03 ppm), 9 (−0.09 ppm), 10 (−0.25 ppm), and 11 (−0.27 ppm) are proposed to originate from PS, SPH, PI, and LPC, respectively. The compound causing signal 12 (−0.38 ppm) is unknown, and this signal is not seen in the spectra of lipids isolated from freshly excised brains. Signal 13 (−0.78 ppm) is tentatively assigned as originating from PC_{AA}, and the signal 14 which is assumed to originate from PC_{DIACYL} is used as the internal reference set at −0.84 ppm.

Accumulation of NAPE in rat brains during post-decapitative ischemia

Accumulation of NAPE subclasses during the observation period of post-decapitative ischemia was followed by ^{31}P NMR spectroscopy as discussed below. The accumulation was additionally investigated by TLC, as shown in **Fig. 3**. The figure shows an accumulation of the NAPE subclasses (the individual subclasses are not separated) during the 6-h period of post-decapitative ischemia.

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. Thus, the spectra were recorded with 60° pulses and the relaxation delay of 1.0 s (the interpulse interval of 1.82 s). Consequently, signals of various phospholipids were saturated to a minor degree (T_1 relaxation times of 1.5 s and 1.3 s determined for PE_{DIACYL} and PE_{PLAS} correspond to the observation of 83% and 86% of the actual signal intensity, respectively). The possible differences in T_1 relaxation times of various phospholipids will therefore lead to slight discrepancies between the relative integrals and molar ratios. Moreover, Lorentz-Gauss transformation was applied to the spectra in order to achieve optimal resolution of the resonances. However, as the T_1 and the apparent transverse (T_2*) relaxation times of the individual phospholipids in different samples are expected to be similar (the total concentration of the phospholipids in the NMR samples was fairly constant), the ratios between the integral intensities of the resonances of a particular compound in different spectra will reflect the molar ratios satisfactorily. To assess the effect of the relaxation delay on the ratio between the integral intensity of the signals of NAPE subclasses and the total integral intensity of all lipid signals, a spectrum of lipids extracted as K-salts from the brain of a 10-day-old rat, exposed to post-decapitative ischemia for 6 h, was recorded with a relaxation delay of 12 s instead of 1 s (total accumulation time over 14.5 h). In this fully relaxed spectrum, the ratio between the integral of the $NAPE_{DIACYL}$ signal and the total lipid signals was identical within experimental error to that observed in the spectrum recorded with the relaxation delay of 1 s. This shows that the effects of the possible variations in T_1 of the individual phospholipids are minor and do not affect the relative integral intensities of the $NAPE_{DIACYL}$ signals in the (almost fully relaxed) spectra recorded with the relaxation delay of 1.0 s to any appreciable degree. Thus, the latter spectra are satisfactory to follow changes in the content of NAPE subclasses in the rat brain lipid extracts by ^{31}P NMR.

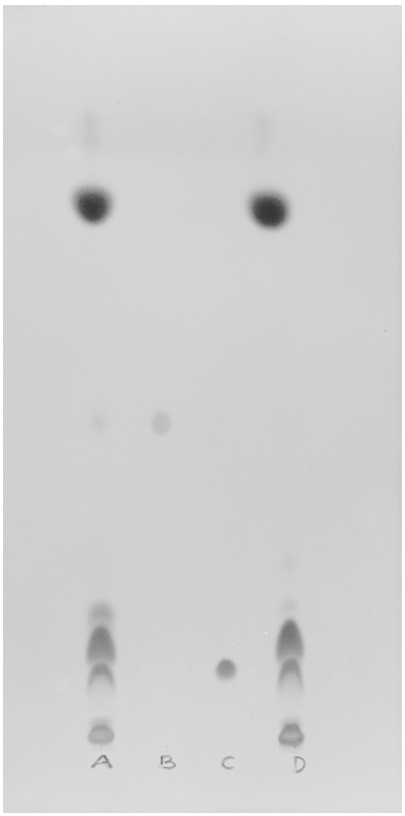

Fig. 3. TLC of rat brain lipids. The plate was eluted with $CHCl_3$–CH_3OH–aq. NH_3 80:20:2 (v/v/v), sprayed with 10% (w/w) copper sulfate in 85% orthophosphoric acid and charred at 200°C. Lane (A): lipid extract of a brain exposed to 6 h of post-decapitative ischemia (0.5 mg); lane (B): $NAPE_{DIACYL}$ standard (10 μg); lane (C): CL standard (10 μg); lane (D): lipid extract prepared immediately after decapitation (0.5 mg).

Figure 4 shows the changes in the integral intensity of $NAPE_{DIACYL}$ resonance as a function of time. The integral intensity of the resonance was expressed in % of the total area of the ^{31}P resonances of all phospholipids in the sample. During the 6 h of post-decapitative ischemia, the relative integral intensity of the $NAPE_{DIACYL}$ signal changed from below 0.09% (detection limit for area determination) to 1.4% (Fig. 4). In addition, the ratio of $NAPE_{PLAS}$/$NAPE_{DIACYL}$ was determined to be 0.53 ± 0.11 (n = 6). This ratio is similar to the ratio of PE_{PLAS}/PE_{DIACYL}, found to be 0.60 ± 0.06 (n = 3). Assuming that the resonance at 0.12 ppm (signal 5) originates from PE_{AA}, the ratio PE_{DIACYL}/PE_{PLAS}/PE_{AA} is (60.2 ± 2.3)/(35.9 ± 2.3)/(3.9 ± 0.2) (n = 3).

After 1 h of post-decapitative ischemia, the formation of $NAPE_{DIACYL}$ amounted to about 0.5% of the total ^{31}P NMR signal (integral intensity). From the observed ratio of $NAPE_{PLAS}$/$NAPE_{DIACYL}$ of 0.53 ± 0.11, the production of total NAPE-subclasses amounts to 0.8% within 1 h and 2%

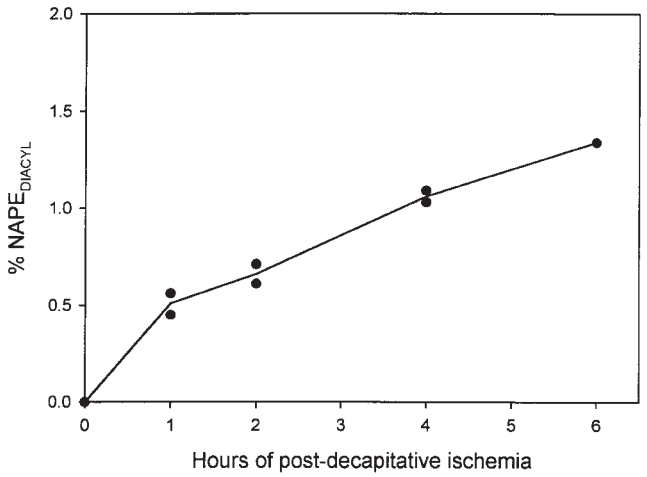

Fig. 4. Post-decapitative ischemia accumulation of $NAPE_{DIACYL}$ in rat brains as measured by ^{31}P NMR spectroscopy of lipid extracts in $CDCl_3$–CH_3OH–H_2O 100:29.9:5.2 (v/v/v). After being decapitated, the heads were incubated at 37°C for various periods of time before the brains were excised, homogenized, and extracted. The areas of the NMR signals of $NAPE_{DIACYL}$ were determined as the percentage of the total area of the phospholipid signals. For each time point, two rat brains were analyzed and the line is drawn through the averages.

within 6 h of post-decapitative ischemia. This result is in agreement with that reported by Natarajan, Schmid, and Schmid (1), who found a linear increase of NAPE subclasses within 60 min of post-decapitative ischemia (at 37°C) in 10-day-old rats up to 0.5% of total phospholipids determined by a chromatographic assay.

Conclusion

NAPE has for many years been known to occur in plant tissue, where its formation has been suggested to serve a cytoprotective role (for a recent paper see ref. 22). It is now generally recognized that this phospholipid is also produced in mammalian tissue, especially in the heart and brain, under pathophysiological conditions such as ischemia (1–3, 23, 24). Under such conditions, the Ca^{2+} level in the cells is elevated, and consequently the enzyme *N*-acyl-transferase, which catalyzes the formation of NAPE by transferring an acyl group from the *sn*-1 position of phospholipids to the amino group of ethanolamine phospholipids, is activated leading to the accumulation of NAPE. Recently, it has been reported that NAPE may be a normal but minor constituent of rat brain lipids (23). Furthermore, NAPE formation may be induced under the condition of glutamate neurotoxicity (25, 26). As glutamate excitotoxicity may be involved in the pathogenic mechanisms of chronic neurodegenerative diseases like Parkinson's disease, Huntington's disease, and Alzheimer's disease (27), the formation of NAPE may be increased in these diseases. The present work demonstrates that ^{31}P NMR spectroscopy can be used for the determination and quantification of NAPE in brain lipids, when the lipids are prepared as K-salts by careful washing of the extracts with K-EDTA. Such determination cannot be carried out with the Cs-salts of lipids, where the NAPE subclass signals coincide with those of PE subclasses. Due to the possible involvement of NAPE in glutamate neurotoxicity and chronic neurodegenerative diseases, ^{31}P NMR spectroscopy may be highly useful in studies of animal models of neurodegenerative diseases. The detection limit of the ^{31}P NMR analysis of NAPE subclasses is subject to substantial improvement by use of improved instrumentation and via extending the time of data accumulation.

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