

Brain lipid metabolism in the cPLA₂ knockout mouse

Thad A. Rosenberger,^{1,*} Nelly E. Villacreses,* Miguel A. Contreras,* Joseph V. Bonventre,[†] and Stanley I. Rapoport*

Brain Physiology and Metabolism Section,* National Institute on Aging, National Institutes of Health, Bethesda, MD 20892; and Massachusetts General Hospital,[†] Harvard Medical School, Charlestown, MA 02129

Abstract We examined brain phospholipid metabolism in mice in which the cytosolic phospholipase A₂ (cPLA₂, Type IV, 85 kDa) was knocked out (cPLA₂^{-/-} mice). Compared with controls, these mice demonstrated altered brain concentrations of several phospholipids, reduced esterified linoleate, arachidonate, and docosahexaenoate in choline glycerophospholipid, and reduced esterified arachidonate in phosphatidylinositol. Unanesthetized cPLA₂^{-/-} mice had reduced rates of incorporation of unlabeled arachidonate from plasma and from the brain arachidonoyl-CoA pool into ethanolamine glycerophospholipid and choline glycerophospholipid, but elevated rates into phosphatidylinositol. These differences corresponded to altered turnover and metabolic loss of esterified brain arachidonate. These results suggest that cPLA₂ is necessary to maintain normal brain concentrations of phospholipids and of their esterified polyunsaturated fatty acids. Reduced esterified arachidonate and docosahexaenoate may account for the resistance of the cPLA₂^{-/-} mouse to middle cerebral artery occlusion, and should influence membrane fluidity, neuroinflammation, signal transduction, and other brain processes.—Rosenberger, T. A., N. E. Villacreses, M. A. Contreras, J. V. Bonventre, and S. I. Rapoport. **Brain lipid metabolism in the cPLA₂ knockout mouse.** *J. Lipid Res.* 2003. 44: 109–117.

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Phospholipases A₂ (EC 3.1.1.4; PLA₂) hydrolyze fatty acids from the *sn*-2 position of phospholipids to form non-esterified fatty acids and lysophospholipids. PLA₂ are classified into eleven types, based on their catalytic activity, amino acid sequence, sequence homology, and mRNA splice variants (1, 2, 3). Types I, II, III, V, IX, X, and XI have low molecular weights, require Ca²⁺ for activity, and have a histidine residue at their catalytic site. Types IV, VI, VII, and VIII have higher molecular weights and are localized in the cell cytosol. These enzymes utilize a serine residue for catalytic activity and may or may not be Ca²⁺-dependent (4). In mammalian brain, mRNA levels indi-

cate that PLA₂ types IIA, IIC, IV, and VI PLA₂ are widely expressed, whereas Type V PLA₂ is expressed at low levels except in the hippocampus (5). The potential of the mammalian brain to modify the expression of the different PLA₂ isoforms provides it with a certain redundancy in the regulation of fatty acid and phospholipid metabolism.

Activation of PLA₂ can release arachidonic acid (AA; 20:4 n-6) or docosahexaenoic acid (DHA; 22:6 n-3) from the *sn*-2 position of membrane phospholipids in brain glia or neurons (4, 6–9). Activation can also produce lysophospholipid activating factor (lysoPAF), which when esterified at its *sn*-2 position becomes PAF, a potent neurotransmitter (10). PLA₂ can be activated via membrane G proteins when certain neurotransmitters bind to specific neuroreceptors, or when Ca²⁺ enters cells following glutamate binding to NMDA receptors or acetylcholine binding to nicotinic receptors (11–15).

Because the different PLA₂ isoforms in brain do not function interchangeably (4), knockout strategies might help to identify their separate roles (16). In this paper, we examine brain lipid metabolism in a mouse in which the 85 kDa type IVA cytosolic PLA₂ (cPLA₂) is absent (cPLA₂^{-/-} mouse) (16–18). cPLA₂ is selective for AA over other fatty acids (1) and requires both Ca²⁺ and phosphorylation for full activation (3, 19). It is involved in neuroreceptor initiated signaling (7, 14), and its transcription is downregulated in rat brain by LiCl administration (7, 14, 20, 21).

The neurological development of the cPLA₂^{-/-} mouse is said to be normal (22). The female knockout mouse has a reduced reproductive ability, whereas adult males are more resistant to middle cerebral artery occlusion than are con-

Abbreviations: AA, arachidonic acid; CerPCho, sphingomyelin; ChoGpl, choline glycerophospholipid; DHA, docosahexaenoic acid; DG, diacylglycerol; EtnGpl, ethanolamine glycerophospholipid; PAF, platelet activating factor; PLA₂, phospholipase A₂; cPLA₂, cytosolic phospholipase A₂; PlsCho, plasmenylcholine; PtdCho, phosphatidylcholine; PlsEtn, plasmenylethanolamine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; PUFA, polyunsaturated fatty acid; sPLA₂, secretory phospholipase A₂; TG, triacylglycerol.

¹To whom correspondence should be addressed.
e-mail: plsetn@mail.nih.gov

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trols (17, 18, 22). The cPLA₂^{-/-} mouse is also more resistant to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopamine depletion (23), and recovers more rapidly from allergen-induced bronchoconstriction. Peritoneal macrophages and mast cells in the cPLA₂^{-/-} mouse fail to produce eicosanoids in response to stimulation (22), suggesting a defective AA cascade (24, 25). Thus, by identifying alterations in brain lipid metabolism in the cPLA₂^{-/-} mouse, we may better understand the role of cPLA₂ in brain structure and function.

MATERIALS AND METHODS

Chemicals

[5,6,8,9,11,12,14,15-³H]Arachidonic acid ([³H]AA, 240 Ci·mmol⁻¹, ≥98% pure) was purchased from Moravak Biochemicals (Brea, CA). Scintillation counting and GC analysis confirmed tracer specific radioactivity. Phospholipid and neutral lipid standards were from Nu-Chek-Prep (Elysian, MN) and "fatty acid free" bovine serum albumin was from Sigma Chemicals (St. Louis, MO). HPLC grade n-hexane and 2-propanol were from EM Science (Gibbstown, NJ). Reagent grade chloroform, methanol, and other chemicals were from Mallinckrodt (Paris, KY) unless noted otherwise. A scintillation cocktail (Ready-Safe, Beckman, Fullerton, CA) containing 1.0% glacial acetic acid was used to determine radioactivity. Extracts were stored in n-hexane/2-propanol (3:2, v/v) + 5.5% H₂O under N₂ at -80°C.

Animals and surgery

Surgery was performed as previously described (26), following the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication no. 80-23), on 3-month-old control (C57Bl/6n) and cPLA₂^{-/-} mice (Charlestown Animal Unit, Massachusetts General Hospital, Boston, MA). Mice were anesthetized with 2-3% halothane (Halocarbon, River Edge, NJ). Polyethylene catheters (PE 10, Becton Dickinson, Sparks, MD) filled with saline containing sodium heparin (100 IU) were implanted into the right femoral artery and vein. The skin was closed and 1% lidocaine was applied as a local anesthetic. The animals were loosely wrapped in a fast-setting plaster body cast, taped to a wooden block, and allowed to recover from anesthesia for 3-4 h. Body temperature was maintained at 36.5°C using a feedback-heating device (Yellow Springs Laboratories, Yellow Springs, OH).

The mouse strains C57Bl/6n and 129/Sv have a naturally occurring defect in the gene encoding the group IIA secretory PLA₂ (sPLA₂), due to a frame shift mutation in exon 3 that results in a T-insertion at position 166 and terminates out of frame in exon 4 (27). The cPLA₂^{-/-} mouse was created using these strains and the 129/Sv cDNA library. Therefore, both the control and cPLA₂^{-/-} mice in this study were deficient in group IIA sPLA₂ (22).

Infusion of [³H]AA

With an infusion pump (Harvard Apparatus, South Natick, MA), unanesthetized mice were infused intravenously for 5 min at a rate of 30 μl·min⁻¹, with 150 μl isotonic saline containing 7.5 mCi·kg body wt⁻¹ [³H]AA suspended in 0.06 mg bovine serum albumin. Arterial samples were collected at fixed times during infusion to determine the radioactivity and concentrations of non-esterified fatty acids and lipids in whole blood and plasma. Five min after starting infusion, mice were killed by an overdose of sodium pentobarbital (100 mg·kg body wt⁻¹, iv) and immediately subjected to head-focused microwave irradiation to stop brain metab-

olism (5.5 kW, 1.2 s; Cober Electronics, Stamford, CT). The brain was excised, frozen on dry ice, and stored at -80°C.

Brain lipid extraction and chromatography

Total lipids from microwaved brains were extracted using n-hexane/2-propanol (3:2, by vol) in a glass Tenbroeck homogenizer (28). Standards and samples in chloroform were applied to Whatman silica gel 60A LK6 TLC plates and separated using chloroform-methanol-acetic acid-H₂O (50:37.5:3:2, v/v/v/v) (29). Bands corresponding to ethanolamine glycerophospholipids (EtnGpl), phosphatidylinositol (PtdIns), phosphatidylserine (PtdSer), choline glycerophospholipids (ChoGpl), and sphingomyelin (CerPCho) were scraped from the TLC plates. The EtnGpl and ChoGpl fractions were extracted from the silica gel using n-hexane/2-propanol (3:2, v/v) + 5.5% H₂O, combined, and concentrated with N₂ at 55°C. They then were subjected to mild acid hydrolysis over hydrochloric acid fumes to cleave the vinyl ether linkage of the plasmalogens. Phosphatidylethanolamine (PtdEtn), lysoplasmeneylethanolamine representing plasmeneylethanolamine (PlsEtn), phosphatidylcholine (PtdCho), and lysoplasmeneylcholine representing plasmeneylcholine (PlsCho) fractions were purified using HPLC (30). Samples were used to quantify fatty acids by GC, radioactivity by liquid scintillation counting, and phospholipid levels by measuring lipid phosphorus (31).

Quantification of labeled and unlabeled acyl-CoA

Acyl-CoA species were isolated from mouse brain extracts using oligonucleotide purification cartridges (Applied Biosystems, Foster City, CA) as previously described (32). Concentrations of acyl-CoA and associated radioactivity were measured using peak area analysis from HPLC chromatograms and liquid scintillation counting. These values were used to calculate the specific radioactivity of arachidonoyl-CoA.

Extraction and separation of plasma

Plasma lipids were extracted by the method of Folch (33). The neutral lipids were separated by TLC on silica gel 60 plates using the solvent system of heptane/diethyl ether/acetic acid (60:40:4, v/v/v) (34). Individual phospholipid classes were separated as previously described. Non-esterified fatty acids and esterified fatty acid in plasma were quantified using gas chromatography, and radioactivity was quantified using liquid scintillation counting.

Tracer identification

Tracer identification and separation was performed on phenacyl ester derivatives of plasma and brain extracted lipids as described previously (35, 36). Phenacyl esters were separated on a LunaTM C18 column (Phenomenex, Torrance, CA) using a linear gradient of acetonitrile and H₂O (20% H₂O at time zero to 8% H₂O over 50 min) on a HPLC (Beckman, Fullerton, CA) equipped with an inline UV/VIS detector (λ = 242 nm, Gilson, Middleton, WI) and inline scintillation counter (β-RAM, IN/US System, Tampa, FL). The phenacyl ester of [³H]arachidonic acid was used to identify tracer radioactivity found in plasma and brain extracts.

Methylation of esterified and non-esterified acids

Esterified fatty acids in samples of the different phospholipid classes were methylated with 0.5 M methanolic potassium hydroxide at 37°C for 30 min. The reaction was stopped with methylformate, and the fatty acid methyl esters were extracted with n-hexane. The non-esterified fatty acids and the esterified fatty acids found in the CerPCho fractions were methylated using 2% sulfuric acid in toluene-methanol (1:1, v/v) at 65°C for 2 h. The reaction was terminated with H₂O and the fatty acid methyl esters were extracted with petroleum ether.

Gas chromatography of fatty acid methyl esters

Fatty acid methyl esters were quantified using a gas chromatograph (Trace 2000, ThermoFinnigan, Houston, TX) equipped with a capillary column (SP 2330; 30 m × 0.32 mm id, Supelco, Bellefonte, PA) and a flame ionization detector. Sample runs were initiated at 90°C with a temperature gradient to 230°C over 20 min. Fatty acid methyl ester standards were used to establish relative retention times and response factors. The internal standard, methyl heptadecanoate, and the individual fatty acids were quantified by peak area analysis. The detector response was linear with correlation coefficients of 0.998 or greater within the sample concentration range for fatty acid standards of differing chain length and degree of saturation.

Calculations

Radioactivity of a brain lipid of interest was calculated by correcting its net brain radioactivity for its intravascular radioactivity (the product of its whole blood radioactivity multiplied by brain blood volume, $2.0 \times 10^{-2} \text{ ml} \cdot \text{g}^{-1}$) (37). Blood samples taken at the time of death (5 min after infusion began) were extracted and analyzed to make this correction.

The model for determining in vivo kinetics of brain fatty acids in rats has been described elsewhere (38, 39). Briefly, unidirectional incorporation coefficients, K_i^* ($\text{ml} \cdot \text{s}^{-1} \cdot \text{g}^{-1}$) of [^3H]AA from plasma into “stable” lipid compartments i were calculated as follows,

$$k_i^* = \frac{c_{br,i}^*(T)}{\int_0^T c_{pl}^* dt} \quad (\text{Eq. 1})$$

$c_{br,i}^*(T)$ ($\text{nCi} \cdot \text{g}^{-1}$) is brain radioactivity of lipid i at time $T = 5$ min (time of termination of experiment), t is time after beginning of infusion, and c_{pl}^* ($\text{nCi} \cdot \text{ml}^{-1}$) is the plasma concentration of labeled AA during infusion. Rates of incorporation of non-esterified AA from plasma into brain phospholipid i , $J_{in,i}$ and from the brain arachidonoyl-CoA into brain phospholipid i , $J_{FA,i}$ were calculated as follows,

$$J_{in,i} = k_i^* c_{pl} \quad (\text{Eq. 2})$$

$$J_{FA,i} = \frac{c_{br,i}^*(T)}{\lambda \int_0^T c_{pl}^* dt} \quad (\text{Eq. 3})$$

C_{pl} ($\text{nmol} \cdot \text{ml}^{-1}$) is the concentration of unlabeled non-esterified AA in plasma. The “dilution factor” λ is defined as the steady-state ratio during [^3H]AA infusion of the specific activity of the brain arachidonoyl-CoA pool to plasma specific activity,

$$\lambda = \frac{c_{arachidonoyl-CoA}^* / c_{arachidonoyl-CoA}}{c_{pl}^* / c_{pl}} \quad (\text{Eq. 4})$$

where the numerator is the ratio of brain arachidonoyl-CoA radioactivity to the unlabeled brain arachidonoyl-CoA concentration. The fractional turnover rate of AA within phospholipid i , $F_{FA,i}$ ($\% \cdot \text{h}^{-1}$), is defined as,

$$F_{FA,i} = \frac{J_{FA,i}}{c_{br,i}} \quad (\text{Eq. 5})$$

The half-life of the FA in i is defined as,

$$\text{Half-life} = 0.693 / F_{FA,i} \quad (\text{Eq. 6})$$

Data and statistics

Integrals of plasma radioactivity were determined by trapezoidal integration, and plasma half-lives were determined by fitting the following equation,

$$c_{pl}^* = c_{pl(t=5)}^* (1 - e^{-\beta t})$$

to plasma radioactivity, where $c_{pl(t=5)}^*$ is steady-state plasma radioactivity and β is a time constant (SigmaPlot, SPSS Science, Chicago, IL). Unpaired Student's t -tests (InStat® Ver. 3.05, GraphPad, San Diego, CA) were used to compare means between $\text{cPLA}_2^{-/-}$ and control mice, where statistical significance was taken as $P \leq 0.05$. Data are presented as mean \pm SD.

RESULTS

Plasma and brain non-esterified fatty acids, and brain acyl-CoAs

There was no statistically significant difference in the mean concentration of any plasma or brain non-esterified fatty acid between $\text{cPLA}_2^{-/-}$ and control mice (Table 1). Additionally, there was no significant difference in the mean brain concentration of any long chain acyl-CoA between the two groups. The ratio of steady-state mean specific activity of arachidonoyl-CoA to plasma AA specific activity, represented by the term λ (Eq. 4), also did not differ significantly between the groups (Table 1). A value for λ equal to 0.04 indicates that about 4% of arachidonoyl-CoA is derived from plasma, with 96% derived by release from phospholipids (38, 39).

Mean arterial plasma radioactivity profiles over time during intravenous [^3H]AA infusion in $\text{cPLA}_2^{-/-}$ and control mice are illustrated in Fig. 1. Steady-state radioactivity, achieved after 180 and 300 s following the start of tracer infusion, equaled $453 \pm 42 \text{ nCi} \cdot \text{nmol}^{-1}$ in $\text{cPLA}_2^{-/-}$ mice and $640 \pm 37 \text{ nCi} \cdot \text{nmol}^{-1}$ in controls ($P < 0.001$). As determined by HPLC analysis of phenacyl ester derivatives in plasma and brain fatty acids from the $\text{cPLA}_2^{-/-}$ (Fig. 2A) and control (data not shown) mice, greater than 97% ($n = 6$) of the radioactivity in plasma and brain extracts was AA at 5 min (Fig. 2B, 2C, respectively). Integrated plasma radioactivity between 0 and 5 min was 27% less in the $\text{cPLA}_2^{-/-}$ than in control mice ($P < 0.01$), consistent with a 35% shorter plasma half-life, $1.05 \pm 0.16 \text{ min}$ compared with $1.61 \pm 0.46 \text{ min}$ in the controls ($P < 0.01$).

Brain phospholipids

Several statistically significant differences were found between $\text{cPLA}_2^{-/-}$ and control mice in mean brain phospholipid concentrations, although the net brain phospholipid concentration did not differ between the two groups (Table 2). Mean concentrations of PtdIns, PtdSer, and CerPCho were increased significantly by 12.5%, 12.8%, and 22.5%, respectively, in the $\text{cPLA}_2^{-/-}$ mice, whereas mean concentrations of EtnGpl and ChoGpl did not differ from control values. Mean brain concentrations each of the PtdEtn, PlsEtn, PtdCho, and PlsCho fractions also did not differ between the groups (data not shown).

TABLE 1. Plasma and brain non-esterified fatty acid, and brain acyl-CoA levels in control and cPLA₂^{-/-} mice

Fatty Acid	Plasma Nonesterified Fatty Acid		Brain Nonesterified Fatty Acid		Brain Acyl-CoA	
	Control	cPLA ₂ ^{-/-}	Control	cPLA ₂ ^{-/-}	Control	cPLA ₂ ^{-/-}
	<i>nmol·ml⁻¹</i>		<i>nmol·g⁻¹</i>		<i>nmol·g⁻¹</i>	
Palmitate (16:0)	80.2 ± 10.5	81.9 ± 10.9	17.0 ± 6.6	19.3 ± 8.1	7.5 ± 0.9	10.2 ± 2.0
Stearate (18:0)	26.8 ± 4.3	24.6 ± 2.8	116.6 ± 9.5	109.4 ± 27.1	5.1 ± 0.3	6.1 ± 0.8
Oleate (18:1n-9)	35.4 ± 9.8	35.7 ± 8.4	18.0 ± 12.5	13.5 ± 4.0	10.9 ± 0.9	12.6 ± 4.1
Linoleate (18:2n-6)	nd	nd	4.0 ± 1.5	3.7 ± 0.9	1.0 ± 0.1	0.9 ± 0.2
α-Linolenate (18:3n-3)	nd	nd	7.8 ± 2.7	8.0 ± 1.9	nd	nd
Arachidonate (20:4n-6)	22.9 ± 5.5	17.9 ± 1.3	9.3 ± 2.1	8.9 ± 1.7	1.7 ± 0.7	1.8 ± 0.2
Docosahexaenoate (22:6n-3)	8.7 ± 3.2	8.1 ± 2.9	11.2 ± 2.0	11.4 ± 2.4	1.7 ± 0.3	1.5 ± 0.3
Total	249.0 ± 38.8	233.2 ± 27.5	309.6 ± 30.5	310.6 ± 67.6	28.7 ± 3.6	30.4 ± 4.5
λ (20:4n-6)					0.040 ± 0.012	0.039 ± 0.020

Values represent the mean ± SD (n = 6). nd, not detected.

Esterified fatty acids

No significant concentration difference was found in any esterified fatty acid within EtnGpl (Table 3). In PtdIns esterified AA was reduced by about 368 nmol·g⁻¹ (35%), whereas esterified stearate was increased by 459 nmol·g⁻¹ and esterified palmitate by 70 nmol·g⁻¹, apparently compensating for the AA decrease. In PtdSer, the only significant difference was a 30% increase in esterified stearate. In ChoGpl, differences were noted in all esterified fatty acids but oleate. Esterified linoleic acid (18:2 n-6) was reduced by about 99 nmol·g⁻¹ (36%), AA by 671 nmol·g⁻¹ (59%), DHA by 724 nmol·g⁻¹ (66%), and stearate by 1,001 nmol·g⁻¹. A compensatory increase of about 2,589 nmol·g⁻¹ was evident in esterified palmitate. The esterified concentration differences in ChoGpl between the cPLA₂^{-/-} and control mouse could be ascribed to differences in PtdCho, as no significant concentration difference was noted in PlsCho (data not shown).

AA incorporation into and turnover within individual brain phospholipids

The significant reduction of esterified AA in brain PtdIns in the cPLA₂^{-/-} mouse was accompanied by an in-

creased rate of incorporation $J_{in,i}$ of AA from plasma (Table 4). This suggests that steady-state loss of AA from brain PtdIns by metabolism was accelerated. The increased $J_{in,i}$ corresponded to increased AA incorporation from the arachidonoyl-CoA pool, $J_{FA,b}$ and a 2-fold increase in AA turnover, $F_{FA,i}$ (Table 5).

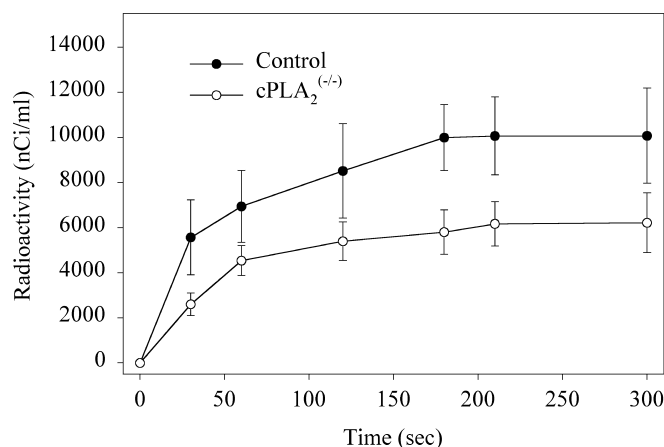


Fig. 1. Time course of plasma radioactivity (nCi·ml⁻¹) in control and cPLA₂^{-/-} mice during intravenous infusion of 7.5 mCi·kg body wt⁻¹ [³H]arachidonic acid over 5 min. Values are the mean ± SD (n = 6).

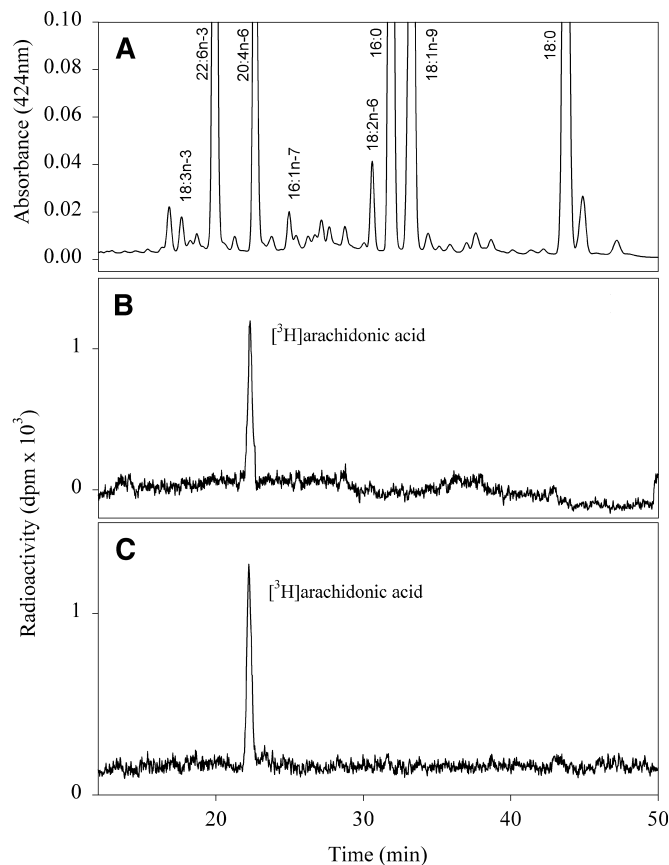


Fig. 2. Representative chromatogram of phenacyl ester derivatives from brain extract (A) and the corresponding radioactivity profiles found in plasma fatty acids (B) and brain fatty acids (C) in cPLA₂ knockout mice infused with 7.5 mCi·kg body wt⁻¹ [³H]arachidonic acid. Abbreviations: 18:3n-3, alpha linolenic acid; 22:6n-3, docosahexaenoic acid; 20:4n-6, arachidonic acid; 16:1n-7, palmitoleic acid; 18:2n-6, linoleic acid; 16:0, palmitic acid; 18:1n-9, oleic acid; 18:0, stearic acid.

TABLE 2. Brain phospholipid levels in control and cPLA₂^{-/-} mice

	Control	cPLA ₂ ^{-/-}
	<i>μmol.g brain⁻¹</i>	
EtnGpl	17.2 ± 1.5	17.0 ± 3.4
PtdIns	2.1 ± 0.2	2.4 ± 0.1 ^a
PtdSer	6.1 ± 0.4	7.0 ± 0.3 ^a
ChoGpl	20.7 ± 2.5	21.7 ± 2.1
CerPCho	3.1 ± 0.1	4.0 ± 0.4 ^a
Total	49.5 ± 4.7	52.2 ± 5.8

EtnGpl, ethanolamine glycerophospholipid; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; ChoGpl, choline glycerophospholipid; CerPCho, sphingomyelin. Values represent mean ± SD (n = 6).

^a *P* < 0.01, differs from control.

Although AA incorporation rates into ChoGpl from plasma and from the arachidonoyl-CoA pool were decreased in the cPLA₂^{-/-} mouse, AA turnover in ChoGpl was elevated, reflecting the disproportionate reduction of esterified AA (Table 5). The increases were found in the PtdCho but not the PlsCho fraction of ChoGpl, in which AA turnover actually was decreased (data not shown). AA incorporation rates into EtnGpl from both plasma and brain arachidonoyl-CoA were reduced about 3-fold in the cPLA₂^{-/-} mouse, consistent with a markedly reduced turnover. The reduction reflected reduced turnover in PtdEtn, as turnover in the PlsEtn fraction was increased (data not shown).

DISCUSSION

Despite marked differences in brain phospholipid and fatty acid composition, and in the kinetics of AA within certain brain phospholipids, the cPLA₂^{-/-} mice demonstrated no significant difference from controls in plasma or brain concentrations of non-esterified fatty acids, brain

levels of acyl-CoA, or the dilution factor λ of brain arachidonoyl-CoA. Thus, measuring only the concentrations of plasma and brain non-esterified fatty acids or of brain acyl-CoA would have provided little evidence of the markedly altered brain lipid composition and AA kinetics in the knockout animal. The only evidence in plasma of altered AA metabolism was a more rapid rate of disappearance of injected [³H]AA than in controls, corresponding to a 35% shorter plasma half-life (Fig. 1).

Our kinetic fatty acid model showed increased rates of incorporation of non-esterified AA from plasma and from brain arachidonoyl-CoA into PtdIns in the cPLA₂^{-/-} mouse (Table 4), and increased turnover of AA within PtdIns and ChoGpl despite the lower concentrations of esterified AA. As *J_{in,i}* represents the rate of replacement by plasma AA of the brain esterified AA that is released and metabolized (40), esterified AA within PtdIns must be hydrolyzed and then metabolized more rapidly than controls. Our study also indicated that AA within ChoGpl turns over at a higher than normal rate in the cPLA₂^{-/-} mouse. Clearly, the absence of cPLA₂ resulted in increased metabolic loss of brain AA, whether by conversion to eicosanoids or by other metabolic pathways (24).

These data suggest that cPLA₂ is involved in the maintenance of esterified AA in PtdIns and ChoGpl. However, the increased incorporation and turnover rates of AA in PtdIns and turnover of ChoGpl in the cPLA₂^{-/-} mice, despite a reduced esterified AA concentration, suggest that other PLA₂ isoforms may compensate for the absent cPLA₂ activity in regulating metabolism of these phospholipid classes. Indeed, cPLA₂ (41), as well as types IIA or V sPLA₂, can augment immediate and delayed AA release in response to cytokines and fetal calf serum (42, 43). The different PLA₂ isoforms can also hydrolyze AA from phospholipid with selectivity for different phospholipid classes (2, 4, 44). Furthermore, other lipolytic enzymes expressed in brain, specifically phosphatidylinositol-specific phos-

TABLE 3. Esterified fatty acid concentrations in brain phospholipids of Control and cPLA₂^{-/-} mice

	EtnGpl		PtdIns	
	Control	cPLA ₂ ^{-/-}	Control	cPLA ₂ ^{-/-}
	<i>nmol × g brain⁻¹</i>			
Palmitate	2,526 ± 323	2,538 ± 472	158 ± 41	88 ± 15 ^b
Stearate	8,352 ± 1,011	9,362 ± 1,335	939 ± 259	1,398 ± 114 ^b
Oleate	5,045 ± 672	4,527 ± 856	155 ± 40	128 ± 22
Linoleate	178 ± 27	154 ± 23	nd	nd
Arachidonate	2,240 ± 571	2,162 ± 469	1,110 ± 297	742 ± 123 ^a
Docosahexaenoate	3,994 ± 1,239	3,213 ± 916	161 ± 36	202 ± 54
	PtdSer		ChoGpl	
Palmitate	256 ± 44	261 ± 57	15,890 ± 2,281	18,479 ± 1,270 ^a
Stearate	6,215 ± 1,060	8,051 ± 1,449 ^a	5,466 ± 650	4,464 ± 377 ^a
Oleate	2,847 ± 585	2,906 ± 926	7,458 ± 1,194	6,365 ± 574
Linoleate	42 ± 5	25 ± 4	275 ± 39	176 ± 33 ^b
Arachidonate	383 ± 61	367 ± 50	1,133 ± 309	462 ± 126 ^b
Docosahexaenoate	4,475 ± 696	4,985 ± 878	1,088 ± 396	364 ± 75 ^b

Values represent mean ± SD (n = 6).

^a *P* < 0.01, differs from control mean.

^b *P* < 0.005, differs from control mean.

TABLE 4. Brain incorporation coefficients, net incorporation rates from plasma non-esterified arachidonic acid, and net incorporation rates from brain arachidonoyl-CoA in control and cPLA₂^{-/-} mice.

	Control		cPLA ₂ ^{-/-}		Control		cPLA ₂ ^{-/-}	
	<i>k_i</i> [*]		<i>J_{m,i}</i>		<i>J_{FA,i}</i>			
	ml·g ⁻¹ ·s ⁻¹ × 10 ⁻⁵		nmol·g ⁻¹ ·s ⁻¹ × 10 ⁻⁴		nmol·g ⁻¹ ·s ⁻¹ × 10 ⁻²			
Triacylglycerol	0.62 ± 0.10	2.28 ± 0.79 ^a	1.41 ± 0.23	5.14 ± 1.77 ^a	0.36 ± 0.06	1.30 ± 0.45 ^a		
Diacylglycerol	0.88 ± 0.28	0.65 ± 0.21	1.98 ± 0.64	1.47 ± 0.48	0.50 ± 0.16	0.37 ± 0.12		
EtnGpl	5.71 ± 1.01	1.75 ± 0.60 ^b	11.68 ± 2.29	3.96 ± 1.35 ^b	2.97 ± 0.58	1.00 ± 0.34 ^b		
PtdIns	6.84 ± 0.75	9.48 ± 1.80 ^a	15.47 ± 1.68	21.42 ± 4.06 ^a	3.93 ± 0.43	5.44 ± 1.03 ^a		
PtdSer	1.78 ± 0.47	1.83 ± 0.62	4.02 ± 1.05	4.14 ± 1.40	1.02 ± 0.27	1.05 ± 0.36		
ChoGpl	5.92 ± 1.44	4.13 ± 0.80 ^a	13.38 ± 3.26	9.33 ± 1.80 ^a	3.40 ± 0.83	2.37 ± 0.46 ^a		
Total	22.62 ± 1.33	19.58 ± 2.55 ^a	51.13 ± 3.01	44.25 ± 4.75 ^a	12.98 ± 0.76	11.23 ± 1.16 ^a		

Values represent mean ± SD (n = 6).

^aP < 0.05, differs from control mean.

^bP < 0.0001, differs from control mean.

pholipase C (45, 46) and phospholipase D (47), can selectively metabolize either PtdIns or PtdCho, respectively. Therefore, redundancy arising because of multiple isoforms of PLA₂ and/or compensation by other lipolytic enzymes expressed in brain might contribute to the increased turnover of brain PtdIns and ChoGpl in cPLA₂^{-/-} mice.

Despite evidence that cPLA₂ is more selective for AA than DHA (1), the absence of cPLA₂ activity throughout life resulted in marked reductions in both esterified AA and DHA. This suggests that the metabolic pathways of each of these two polyunsaturated fatty acids (PUFAs) are interactive and in part regulated by cPLA₂. For example, inhibition by DHA of the conversion of AA to eicosanoids by cyclooxygenase-2 or 5-lipoxygenase has been reported (48, 49). Competition between n-6 and n-3 PUFA acyl-CoA can also occur at the level of desaturation and elongation (50–52), in that during nutritional n-3 deprivation, brain AA will be converted to eicosapentaenoic acid (22:5 n-6) (53). However, the absence of esterified 22:5n-6 due to the lack of retrograde conversion or acyl chain elongation of [³H]AA in the brain of cPLA₂^{-/-} (Fig. 2) and control mice (data not shown) are consistent with normal concentrations of brain acyl-CoA and plasma non-esterified fatty acid in the cPLA₂^{-/-} mouse (Table 1).

Thus, the interaction between AA and DHA metabolism appears not to occur at the level of recycling within brain phospholipids. In this regard, rats subjected to nutritional n-3 PUFA deprivation for three generations have an reduced turnover of DHA but unaffected AA turnover within brain phospholipids (53), whereas chronic administration of LiCl to rats reduces AA turnover without affecting DHA turnover (54, 55). The independent recycling of AA and DHA within phospholipids is consistent with the presence of an AA-specific PLA₂ (1, 56, 57) and an AA-specific arachidonoyl-CoA synthetase (58, 59). In the cPLA₂^{-/-} mouse, on the other hand, compensation from other phospholipid-metabolizing enzymes with differing acyl-chain specificity may account for the observed changes in both AA and DHA composition. Further studies are required to investigate the effects that the absence of cPLA₂ has on the activity and expression of these enzymes, and on their ability to maintain plasma non-esterified fatty acid and lambda AA-CoA at control levels de-

spite the apparent reduction in the plasma half-life of [³H]AA and the altered turnover rates of brain phospholipid. Additionally, we should determine incorporation and turnover rates of DHA in brain phospholipids of the cPLA₂^{-/-} mouse. The question remains as to what extent the alterations found in the knockout mouse are due to the lack of cPLA₂, compared with compensatory effects by other enzymes.

The marked reductions in the cPLA₂^{-/-} mouse of esterified linoleic acid, AA, and DHA in brain ChoGpl and of AA in PtdIns, and abnormalities in brain phospholipid composition, would be expected to alter brain function and structure (60). Although neurological development is said to be normal in the cPLA₂^{-/-} mouse (22), sophisticated memory and behavioral tests have not been performed on these animals. In the absence of altered brain function, the involvement of cPLA₂ and the relevance of the observed alterations in brain phospholipid metabolism to normal brain physiology are not clear. The apparent increase in the rate of incorporation and turnover of AA in PtdIns and increased turnover of ChoGpl, suggest that there are compensatory changes in other phospholipid-metabolizing enzymes. However, the reported resistance of the knockout mouse to middle cerebral artery occlusion (17, 18) may arise from reduced availability and release of esterified AA during insult as a result of the absence of cPLA₂ activity (61–63). This interpretation is consistent with evidence that chronically administered LiCl in rats increases their resistance to cerebral ischemia (64), while decreasing brain cPLA₂ expression and AA turnover (21, 54).

TABLE 5. Turnover rates of arachidonic acid in brain phospholipids of control and cPLA₂^{-/-} mice

	Control		cPLA ₂ ^{-/-}	
	<i>F_{FA,i}</i>			
	%·h ⁻¹			
EtnGpl	4.8 ± 0.9		1.7 ± 0.6 ^a	
PtdIns	12.7 ± 1.4		26.4 ± 5.0 ^a	
PtdSer	9.6 ± 2.5		10.3 ± 3.5	
ChoGpl	10.8 ± 2.6		18.5 ± 3.6 ^a	

Values represent the mean ± SD (n = 6).

^aP < 0.005.

Differences in esterified brain PUFA concentrations in the cPLA₂^{-/-} mouse also would be expected to alter brain membrane fluidity, receptor function, membrane remodeling, neuroplasticity, and resistance to apoptosis, among other processes (10, 65, 66). The absence of cPLA₂ can affect receptor-initiated signaling processes in which it participates to release AA and initiate the AA cascade (7, 14, 24, 25). The reported absence of eicosanoid formation in stimulated macrophages and mast cells from the cPLA₂^{-/-} (22) is consistent with this prediction. Alterations in the AA cascade may also account for the reduced female fertility in the cPLA₂^{-/-} mouse (18), as fetoplacental development involves cPLA₂ activation and prostaglandin formation (67). Therefore, the reported resistance of the cPLA₂^{-/-} mouse to middle cerebral artery occlusion, absence of eicosanoid formation, and reduced female fertility rates can be attributed to the loss of cPLA₂ activity. However, whether the quantitative contribution of the observed changes in phospholipid metabolism found in these studies are due to the primary knockout or to secondary compensatory mechanisms is at this point unclear. Further studies measuring the changes in behavior, turnover of other brain fatty acids including DHA, and expression patterns of those enzymes associated with PtdIns and PtdCho metabolism will help us better understand the role that cPLA₂ has in brain function.

This paper extends our kinetic fatty acid method, developed in unanesthetized rats (38, 39) to unanesthetized mice. We found that the brain concentrations of phospholipids, esterified and non-esterified fatty acids, and acyl-CoA species in control mice were comparable to the respective concentrations reported in rats (53, 68). Derived kinetic parameters for AA were also comparable, with the exception of AA turnover within EtnGpl (53, 69). Control AA turnover within each of the phospholipid classes ranged from 4.8%·h⁻¹ (EtnGpl) to 1.3%·h⁻¹ (PtdIns) in the mouse brain (Table 4), equivalent to half-lives (Eq. 6) of 5.5 h (PtdIns) to 14 h (EtnGpl). In rat brain, turnover rates of 9.4·h⁻¹, 6.0·h⁻¹, 5.4·h⁻¹, and 0.3%·h⁻¹ have been reported for ChoGpl, PtdSer, PtdIns, and EtnGpl, respectively. In both species, calculated half-lives of hours are many fold shorter than half-lives days or weeks found when fatty acid recycling with brain phospholipids was ignored (38, 70, 71).

The 10-fold higher AA turnover in EtnGpl in control mice than in control rats may represent a species difference, as the distribution of radioactivity in brain EtnGpl and ChoGpl following the intracerebral injection of [¹⁴C]arachidonic acid (72) was similar to ours following intravenous injection. Alternatively, the difference may reflect that our control mice were deficient in group IIA sPLA₂ (22, 27), since phosphatidylethanolamine may be the preferred phospholipid substrate for sPLA₂ (73, 74). Another possibility is that the difference reflected a smaller fraction of white matter myelin in the mouse brain compared to the rat brain, and a more rapid turnover of EtnGpl in gray than white matter (68, 75).

In summary, a knockout animal may not provide a clear understanding of the normal role of a specific gene product, as its phenotype can result from primary gene loss as

well as secondary changes during development and maturation (16). In this regard, a 50% reduction in brain cPLA₂ transcription in the adult rat, caused by 6 weeks of LiCl administration, did not alter esterified brain concentrations of either AA or DHA, while reducing AA but not DHA turnover in brain phospholipids (20, 21, 54) and suggests that a conditional cPLA₂ knockout, when available, might better elucidate the role of cPLA₂ in brain than the lifetime knockout (16). Nevertheless, the absence of cPLA₂ throughout development and maturation results in multiple changes involving brain membrane phospholipid composition, reductions in the levels of brain esterified AA and DHA, and alterations in the kinetics of brain AA metabolism. Finally, these results identify multiple ways in which the cPLA₂^{-/-} phenotype can be further examined so as to better understand how cPLA₂ regulates brain structure, modulates signal transduction, as well as aid in future studies investigating its role in mechanisms associated with injury. ■

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