Biochemical Pharmacology, Vol. 55, pp. 515–521, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00501-7

Effect of Chronic Ethanol Exposure on Mouse Brain Arachidonic Acid Specific Phospholipase A_2

Balapal S. Basavarajappa, Thomas B. Cooper*† and Basalingappa L. Hungund*†‡*

*Division of Analytical Psychopharmacology, New York State Psychiatric Institute, and †Department of Psychiatry, College of Physicians and Surgeons, Columbia University, New York, NY 10032, U.S.A.

ABSTRACT. The enzyme phospholipase A_2 (PLA_2), which catalyzes the hydrolysis of an ester bond at the *sn*-2 position of 1,2-*sn*-diacylglycerols, has been suggested to play an important role in regulating cellular functions. Although ethanol (EtOH)-induced activation of PLA_2 activity was reported previously by us in mouse brain (Hungund *et al.*, *Neurochem Int* **25:** 321–325, 1994), its subcellular localization and biochemical properties have not been investigated. Therefore, in the present study, we examined the subcellular localization and characterization of EtOH-activated PLA_2 activity in mouse brain. The results indicated that EtOH treatment decreased the specific activity of PLA_2 for the first 48 hr, and then the activity increased and reached a peak level in both cytosol (1.6-fold) and membrane (1.7-fold) fractions at 96 hr of exposure. Specific activity was found to be higher in the membrane fraction than in the cytosol. Using differential density gradient centrifugation, subcellular localization of the membrane-associated PLA_2 revealed that most of the EtOH-activated PLA_2 specific activity was associated with the synaptic membrane (44%) followed by the nuclear membrane (13%). No significant increase in the PLA_2 specific activity of mitochondrial and microsomal membranes was observed. No activity was detected in the myelin membrane. PLA_2 specific activity of membranes from control and EtOH-exposed mouse brain exhibited preference for arachidonic acid over linoleic acid at the *sn*-2 position of glycero-3-phosphocholine (PC). No detectable PLA_2 specific activity was found when PC containing oleic acid at the *sn*-2 position was used as a substrate. The present results also indicated that the PLA_2 specific activity of membrane from control and EtOH-exposed mouse brain was insensitive to dithiothreitol, strongly stimulated by Ca^{2+}, enhanced by glycerol, and inhibited by the cytosolic PLA_2 ($cPLA_2$) inhibitor methyl arachidonyl fluorophosphonate with an IC_{50} value of 3.33 μM. In summary, results suggest that the properties of EtOH-activated PLA_2 activity found in mouse brain membrane fraction are similar to those of $cPLA_2$ found in variety of cells, including mammalian brain. BIOCHEM PHARMACOL **55;4:515–521, 1998.** © 1998 Elsevier Science Inc.

KEY WORDS. brain; ethanol; PLA_2; Ca^{2+}; MAFP

Chronic EtOH§ has been shown to produce alterations in neuronal plasma membrane function [1–6]. However, the molecular mechanisms that underlie these effects have not been identified. There is overwhelming evidence that EtOH exerts its pharmacological effects by modulating the function of many components of intracellular signal transduction pathways [7–9], in addition to its action on fatty acyl composition of cell membrane phospholipids [10, 11]. PLA_2 (EC 3.1.1.4), an enzyme that hydrolyzes the *sn*-2 fatty acyl ester bond of phosphoglycerides, has been shown to be involved in the regulation of phospholipid acyl turnover for membrane repair or the production of inflammatory lipid mediators [12]. Mammalian cells contain structurally diverse forms of PLA_2 including $sPLA_2$ (14 kDa), $cPLA_2$ (85 kDa) and cytosolic $iPLA_2$ [13–15]. $cPLA_2$, which preferentially hydrolyzes *sn*-2 arachidonic acid, shares no homology with other PLA_2 enzymes. Although $sPLA_2$ and $iPLA_2$ enzymes do not exhibit acyl chain specificity, they can also mediate arachidonic acid release, depending on the cell type and agonist involved [12]. EtOH-induced release of arachidonic acid and its metabolites is implicated to play a significant role in the mediation of important cellular events, including signal transduction [16]. PLA_2 has been shown to increase by EtOH treatment in *in vivo* [17, 18] and *in vitro* [19] systems, and in chick embryo [20] and in mouse peritoneal macrophage [21] models. This increase in PLA_2 activity has been suggested to reduce the proportion of unsaturated acyl composition of selected membrane phospholipids and thus influence the development of resistance to the disordering effects of EtOH [13, 14]. The presence of diverse PLA_2 enzymes in mammalian cells makes it difficult to understand the role of each of these enzymes in the

‡ Corresponding author: B. L. Hungund, Ph.D., NYS Psychiatric Institute at NKI, Bldg. #37, 140 Old Orangeburg Road, Orangeburg, NY 10962. Tel. (914) 365-2000, Ext. 1327; FAX (914) 398-0378.

§ *Abbreviations*: $cPLA_2$, cytosolic PLA_2; DTT, dithiothreitol; EtOH, ethanol; $iPLA_2$, Ca^{2+}-independent PLA_2; MAFP, methyl arachidonyl fluorophosphonate; $sPLA_2$, secretory PLA_2; PLA_2, phospholipase A_2; and PMSF, phenylmethylsulfonyl fluoride.

Received 8 May 1997; accepted 18 August 1997.

important processes of fatty acid turnover in EtOH tolerance and dependence. Hence, the present study was undertaken to characterize and determine the subcellular localization and the type of PLA_2 involved in the chronic effects of EtOH, using a mouse inhalation model [18, 22, 23]. The results of this study suggest that chronic EtOH exposure specifically activated Ca^{2+}-dependent, DTT-insensitive PLA_2 enzyme, which hydrolyzes *sn*-2-arachidonic acid from *sn*-glycero-3-phosphocholine preferentially compared with 2-linoleoyl or oleoyl-*sn*-glycero-3-phosphocholine. The majority of the arachidonic acid specific PLA_2 specific activity was associated with the synaptic membrane fraction.

MATERIALS AND METHODS

Male Swiss–Webster mice (25–30 g, 6 to 8-weeks-old) were purchased from Taconic Farms. $cPLA_2$ inhibitor (MAFP) was purchased from Cayman Chemicals. HPTLC plates were from VWR Scientific. Liquid scintillation fluid was obtained from National Diagnostics. Unlabeled phospholipids and fatty acids were purchased from Avanti Polar Lipids. All radiolabeled phospholipids [1-palmitoyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine (55 mCi/mmol), 1-palmitoyl-2-[1-^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine (55 mCi/mmol), and 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (55 mCi/mmol)] were purchased from American Radiolabeled Chemicals Inc. All other chemicals were obtained from the Sigma Chemical Co.

Animals and EtOH Administration

Animals were exposed chronically to EtOH by an inhalation procedure for periods of up to 4 days [18, 22, 23]. An i.p. injection of pyrazole (68 mg/kg) was given daily to maintain a relatively constant blood EtOH level. Control animals similarly treated were housed under identical conditions except for the absence of EtOH in the inspired air. Blood EtOH levels were determined using an enzymatic method [24]. Animals were decapitated, and brains were removed and processed immediately for the preparation of cellular fractions.

Preparation of Subcellular Fractions

The brains were removed, transferred quickly into 3 vol. of cold buffer containing 10 mM Tris–HCl (pH 7.4), 320 mM sucrose, freshly added protease inhibitors, 0.1 mM PMSF, 20 μM leupeptin, and 5 μM pepstatin (buffer A), and homogenized in a motor-driven Potter–Elvehjem homogenizer fitted with a Teflon pestle. All procedures were conducted at 4°. The homogenate was centrifuged at 900 × *g* for 20 min. The pellet, which contained nuclei, was resuspended in 4.5 vol. of buffer A and centrifuged at 800 × *g* for 20 min, and all contaminants were thoroughly washed out essentially as described [25]. The nuclear membrane was suspended in 10 mM Tris–HCl (pH 7.4) and stored at −70° until used. The supernatant was centrifuged at 100,000 × *g* for 60 min at 4°. The resultant supernatant represented the cytosolic fraction (S100) and the pellet represented the membrane fraction (P100).

The membrane fraction (P100) was resuspended in a buffer containing 10 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, and 1 M KCl (pH 7.4) and incubated for 1 hr at 4° to establish whether the PLA_2 activity found with the membrane fraction (P100, total) was membrane bound or associated. The mixture was then centrifuged at 100,000 × *g* for 30 min, and PLA_2 activity in the supernatant and pellet was determined. The recovery of membrane PLA_2 in the supernatant was greater than 90% of the activity found in the initial pellet suspension.

Preparation of Mouse Brain Membrane Fractions

Various membrane fractions were prepared by following established methods [26, 27]. Tissue was suspended in 9 vol. of buffer B [50 mM Tris–HCl (pH 7.4), 10% sucrose containing freshly added protease inhibitors, 0.1 mM PMSF, 20 μM leupeptin, and 5 μM pepstatin]. All procedures were conducted at 4° with pre-cooled solutions. After homogenization with 20 strokes of a Teflon pestle, the homogenate was centrifuged at 800 × *g* for 20 min. The supernatant was removed, rehomogenized, and centrifuged at 16,000 × *g* for 30 min. This procedure was repeated one more time. The pellet (16,000 × *g*) was resuspended in 4.5 vol. of a hypotonic buffer [5 mM Tris–HCl, (pH 8.1) and freshly added protease inhibitors, 0.1 mM PMSF, 20 μM leupeptin, and 5 μM pepstatin] and gently homogenized with three strokes of a Teflon pestle. The homogenate was incubated for 30 min at 4° to lyse the synaptosomes and vesicles. The incubation mixture was homogenized with 10 strokes of a Teflon pestle. The homogenate was supplemented with sucrose to yield a 34% sucrose (w/w) suspension in 50 mM Tris–HCl, (pH 7.4) and was layered at the bottom of a three-step gradient. An equal volume of 28.5% sucrose/Tris–HCl (w/w) and a one-third volume of 10% sucrose Tris–HCl (w/w) were overlaid carefully on the top of the 34% sucrose (w/w) suspension. The gradients were centrifuged at 60,000 × *g* for 2 hr and the separated myelin, synaptosomal, and mitochondrial membranes were resuspended separately in 10 mM Tris–HCl, (pH 7.4), diluted to 10% sucrose, and pelleted by centrifugation at 40,000 × *g* for 30 min. Synaptosomal membrane purity was assessed using lactate dehydogenase [28] and Na^+,-K^+-ATPase enzyme markers [29].

The supernatant (16,000 × *g* for 30 min) was further centrifuged at 100,00 × *g* for 60 min to prepare a microsomal membrane. Each membrane fraction was resuspended in 10 mM Tris–HCl buffer (pH 7.4), freshly added protease inhibitors, 0.1 mM PMSF, 20 μM leupeptin, and 5 μM pepstatin, and stored at −70°.

PLA_2 *Assay*

PLA_2 activity was measured using the standard reaction mixture (500 μL) containing 20 μM 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine containing 1-palmitoyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine (1×10^5 dpm), 20 mM glycine buffer (pH 8.0), 5 mM $CaCl_2$, and 0.1% Triton X-100, which was vortexed thoroughly [30]. Then the suspension was sonicated for 1 min at 37°, and the reaction was initiated by adding the enzyme preparation (600 μg protein). The incubation was carried out at 37° for 30 min, and the reaction was terminated by adding 2 mL of Dole's reagent [31]. The released free [^{14}C]arachidonic acid was extracted [30], and the radioactivity in the extract was determined. PLA_2 activity was expressed as picomoles of arachidonic acid released per hour per milligram of protein. All the assays were carried out in triplicate. Values were corrected for nonenzymatic hydrolysis (no enzyme). Preliminary experiments were conducted to establish kinetic parameters of the enzyme by varying concentrations of substrate (0–100 μM), amounts of tissue (100–800 μg protein), and periods of incubation (0–60 min). The present conditions of the assay were within a linear relationship with respect to concentrations of substrates, amount of tissue protein, and incubation time.

Protein Determination

Protein concentration of the subcellular fractions was determined by the procedure of Lowry *et al.* [32], using bovine serum albumin as the standard.

Statistical Analysis

Student's *t*-test was used to evaluate statistical comparisons. Differences were considered to be significant at $P < 0.05$. Data are presented as means ± SEM from at least three separate experiments run in triplicate, unless otherwise indicated.

RESULTS

The blood EtOH levels after 24 hr of EtOH exposure reached a mean value of 2.7 ± 0.03 mg/mL and then remained stable for up to 96 hr of exposure. Exposure of mice to EtOH for 96 hr had no significant effect on either body or brain weight.

The effect of EtOH on PLA_2 activity was measured initially in cytosol and membrane fractions prepared after various periods of EtOH exposure, using 1-palmitoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine as a substrate (Fig. 1). The results suggest that initially the PLA_2 specific activity in both the cytosol and membrane fractions decreased for up to 48 hr of EtOH exposure and then progressively increased significantly, reaching a higher level thereafter (72–96 hr). The results indicate that both the cytosol and membrane fractions contained a significant amount of PLA_2 specific activity; it was found to be highest in the membrane fraction from EtOH-exposed mouse brain when compared with the control (Table 1). The membrane fraction that had the highest PLA_2 specific activity was used for further characterization.

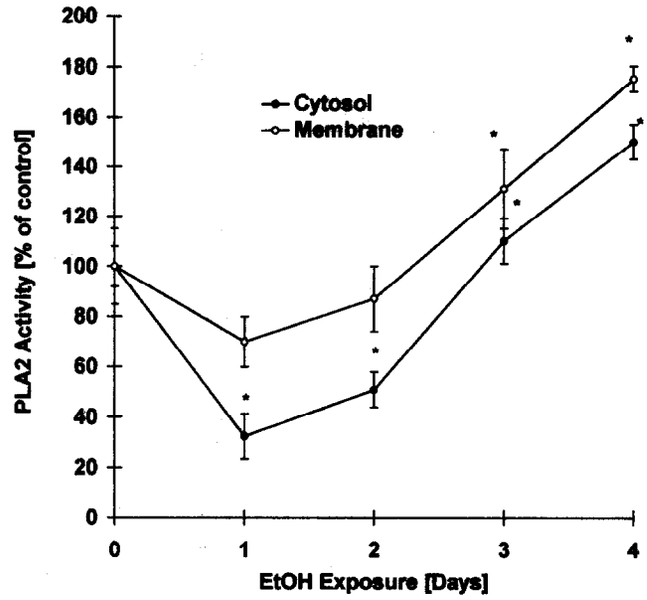

FIG. 1. Changes in the specific activity of PLA_2 in mouse brain following exposure to EtOH for various periods of time. Each assay (500 μL) containing 20 μM 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine (1×10^5 dpm), 20 mM glycine-buffer (pH 8.0), 5 mM $CaCl_2$, and 0.1% Triton X-100 was incubated with membrane or cytosol fraction (600 μg protein) for 30 min at 37°. Results are expressed as picomoles of fatty acid released per hour per milligram of protein. Values shown are the means ± SEM of three separate experiments done in triplicate. Control values for all time points were 70.92 ± 3.0 pmol/hr/mg protein for cytosol and 232.8 ± 60 for membrane. Key: (*) $P < 0.05$, compared with control.

TABLE 1. PLA_2 activity in subcellular fractions of EtOH-exposed (4 days) mouse brain

Fractions	PLA_2 activity (pmol/hr/mg protein)	
	Control	EtOH
Particulate (P100)	232.8 ± 60.0	392 ± 30.0*
Soluble (S100)	70.92 ± 3.0	113.85 ± 22.8*
Nuclear	34.50 ± 1.62	81.78 ± 6.78*
Synaptic	98.64 ± 6.60	173.58 ± 8.28*
Mitochondrial	66.66 ± 1.98	73.14 ± 3.12
Microsomal	66.60 ± 4.74	69.93 ± 8.94

PLA_2 of each membrane fraction (600 μg protein) was assayed under standard conditions using 20 μM 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine containing 1-palmitoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine (1×10^5 dpm), 20 mM glycine buffer (pH 8.0), 5 mM $CaCl_2$, and 0.1% Triton X-100. The values shown are picomoles of fatty acid released per hour per milligram of protein (means ± SEM) from three separate experiments done in triplicate.

* $P < 0.05$, compared with control.

Subcellular Localization of PLA_2 from Mouse Brain

Studies were extended to determine the localization of EtOH-activated PLA_2 among various membrane fractions prepared by the sucrose density gradient method. Table 1 shows that the EtOH-activated PLA_2 specific activity was localized in the synaptic membrane (44%) and was followed by the nuclear membrane (13%) in comparison with the specific activity found in the total membrane fraction. No significant increase in the PLA_2 activity was observed in mitochondrial and microsomal membranes of EtOH-exposed brains. No PLA_2 activity was found in the myelin membrane fraction.

Control and EtOH-exposed mouse brain membrane fractions (P100, total) were treated with 1 M KCl to determine whether the PLA_2 activity found in the membrane fraction (P100, total) was membrane bound or associated. A large percentage (>90%) of the membrane PLA_2 specific activity was solubilized with 1 M KCl, suggesting that the PLA_2 specific activity was membrane associated rather than membrane bound (data not shown).

Evaluation of Ca^{2+} and pH Dependence of EtOH-Activated Membrane-Associated PLA_2

The effect of free Ca^{2+} on the enzymatic activity was examined with Ca^{2+}/EGTA buffers to accurately maintain the free Ca^{2+} levels [33]. The PLA_2 activity of the membrane from EtOH-treated mouse brain was found to be dependent upon the presence of Ca^{2+}. The basal activity of the particulate enzyme, although not very active in the absence of added calcium, was stimulated by the addition of Ca^{2+} and exhibited a concentration–response curve with maximal activity at millimolar calcium concentrations but with significant activity detected at low Ca^{2+} concentration. Most of the enzyme activation occurred at less than 500 μM Ca^{2+} in membrane from control mouse brain; a significant increase in the PLA_2 specific activity was observed above this concentration only in EtOH-exposed mouse brain (Fig. 2).

The pH dependence of the PLA_2 activity of the membrane fractions from control and EtOH-exposed mouse brain was studied. The pH-activity profile of both the membrane fractions was in the range between pH 6 and 9 and possessed an alkaline pH optimum of ~8.5. A detectable amount of PLA_2 specific activity was observed in the membrane fractions from control and EtOH-treated tissue at pH 6.0 (data not shown).

Effect of Glycerol on the Activity of EtOH-Activated Membrane-Associated PLA_2

Glycerol increases the $cPLA_2$-catalyzed hydrolysis of phospholipids [34–36]. An increase in the concentration of glycerol in the assay from 0 to 60% (by volume) increased the rate of the hydrolysis of 1-palmitoyl-2-[1-^{14}C]arachidonyl *sn*-glycero-3-phosphocholine. The maximum PLA_2 specific activity of the membrane from control (2.3-fold) and EtOH-treated (1.5-fold) mouse brain was observed at 20% glycerol, and no further increase was observed above this concentration (data not shown).

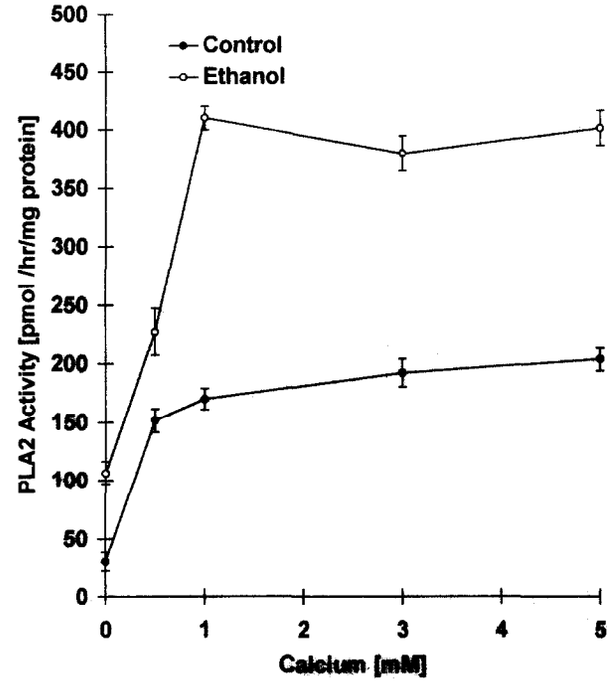

FIG. 2. Effect of Ca^{2+} on the specific activity of PLA_2 in membrane fractions from EtOH-treated (4 days) mouse brain. Each assay (500 μL) containing 20 μM 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine (1 × 10^5 dpm), 20 mM glycine buffer (pH 8.0), and 0.1% Triton X-100 with indicated concentrations of Ca^{2+} in a Ca^{2+}/EGTA buffer system was incubated with membrane fraction (600 μg protein) for 30 min at 37°. Results are expressed as picomoles of fatty acid released per hour per milligram of protein. Values shown are the means ± SEM of three separate experiments done in triplicate.

Effect of $cPLA_2$ Inhibitor (MAFP) on the EtOH-Activated Membrane-Associated PLA_2

MAFP is an irreversible inhibitor of $cPLA_2$ but has no effect on $sPLA_2$ [37]. MAFP was used to determine the type of PLA_2 activated by EtOH in mouse brain. Figure 3 shows that MAFP strongly inhibited both the control and EtOH-activated PLA_2 activity of the membrane fraction with an IC_{50} value of 3.33 μM.

Substrate Preference of EtOH-Activated Membrane PLA_2

The ability of the PLA_2 from EtOH-treated mouse brain membrane fraction to catalyze the hydrolysis of different phosphoglyceride substrates is shown in Table 2. The highest rate of the hydrolysis was observed using 1-palmitoyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine as a substrate (3.4-fold higher) compared with 1-palmitoyl-2-[1-^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine (2.1-fold).

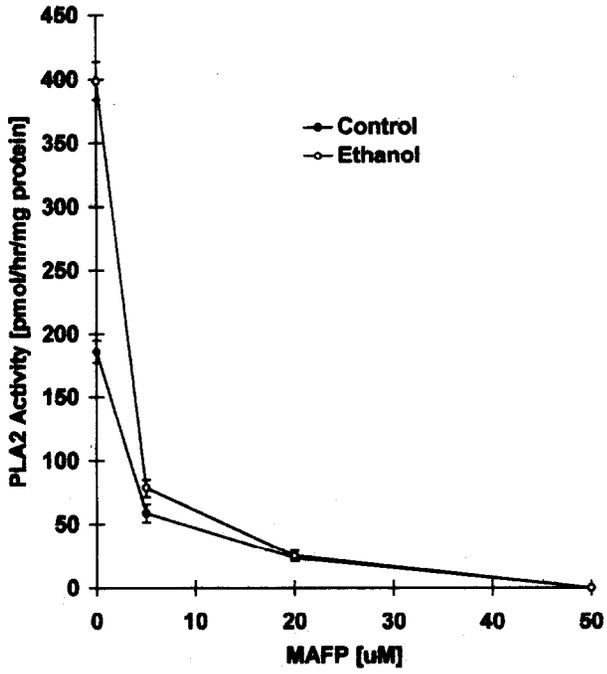

FIG. 3. Effect of $cPLA_2$ inhibitor (MAFP) on the activity of membrane fraction from EtOH-exposed (4 days) mouse brain. Each assay (500 μL) containing 20 μM 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine (1×10^5 dpm), 20 mM glycine buffer (pH 8.0), 5 mM $CaCl_2$, and 0.1% Triton X-100 with indicated concentrations of MAFP (μM) was incubated with membrane fraction (600 μg protein) for 30 min at 37°. PLA_2 activity was expressed as picomoles of fatty acid released per hour per milligram of protein. Values shown are the means ± SEM of three separate experiments.

No detectable PLA_2 activity was found in the control and EtOH-treated membrane fractions when 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine was used as a substrate. The order of substrate specificity for the enzymatic hydrolysis in the both control and EtOH-treated membranes was *sn*-2-arachidonoyl > linoleoyl > oleoyl glycero-3-phosphocholine.

TABLE 2. Substrate specificity of EtOH-activated (4 days) mouse brain membrane PLA_2

	PLA_2 activity (pmol/hr/mg protein)	
Various substrates	Control	EtOH
1-pam-2-Δ_4Ach-ptdCho	232.8 ± 3.0	398 ± 30.0*
1-pam-2-$\Delta_2$lin-ptdCho	39.12 ± 3.06	83.34 ± 5.76*
1-pam-2-$\Delta_1$ole-ptdCho	0.0	0.0

Each PLA_2 assay (500 μL) containing 20 μM 1-palmitoyl-2-acyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-[^{14}C]acyl-*sn*-glycero-3-phosphocholine (1×10^5 dpm), 20 mM glycine buffer (pH 8.0), 5 mM $CaCl_2$, and 0.1% Triton X-100 was incubated with membrane fraction (600 μg) for 30 min at 37°. Each 1-palmitoyl-2-[^{14}C]acyl-*sn*-glycero-3-phosphocholine was radiolabeled at the *sn*-2 position with different fatty acids. Results are expressed as picomoles of fatty acid formed per hour per milligram of protein (mean ± SEM) from three separate experiments done in triplicate. Abbreviations: pam, palmitoyl; ptdCho, phosphatidylcholine; Ach, arachidonyl; lin, linoleoyl; and ole, oleoyl.

* $P < 0.05$, compared with control.

Effect of DTT on the PLA_2 Activity of Membrane Fraction

Treatment with sulfhydral reducing agents, such as DTT, has been reported to denature the disulfide bridges contained in low molecular weight PLA_2 ($sPLA_2$) [38]. The PLA_2 specific activity in membrane fractions from control and EtOH-treated tissues was not altered significantly by pretreatment with 4 or 10 mM DTT incubated at 37° for 30 min (data not shown).

DISCUSSION

The mouse inhalation model has been used for investigating the cellular and molecular events that underlie EtOH intoxication, tolerance [18, 39], and withdrawal [22, 23]. We have reported previously that chronic EtOH exposure increases PLA_2 activity in mouse brain [18]. In the current study, we investigated the biochemical properties and subcellular distribution of EtOH-activated PLA_2 enzyme.

The present results of EtOH-induced activation of PLA_2 activity in mouse brain concur with the results previously reported in *in vivo* systems [17, 18], and in chick embryo [20] and mouse peritoneal macrophage [21] models. For the first time we have shown here that EtOH treatment leads to activation of a PLA_2 that preferentially hydrolyzes 2-arachidonyl-*sn*-glycero-3-phosphocholine compared with 2-linoleoyl or oleoyl-*sn*-glycero-3-phosphocholine as a substrate. Most of this arachidonic acid specific PLA_2 activity was localized in the membrane fraction. Similar arachidonoyl-specific PLA_2s have been shown to exist in a variety of stimulated cells [39–42], rat platelets [43], rat brain [44], and human brain [45] and have been shown to translocate to membrane in a Ca^{2+}-dependent manner [42–44]. It is interesting to note that the observed decrease in specific activity at 24 and 48 hr of EtOH exposure (75 and 50% of control values) reflects the short-term effect of EtOH. The subsequent increase in PLA_2 specific activity with increasing periods of EtOH exposure (72–96 hr) may be an adaptation mechanism to the continuous presence of EtOH.

The PLA_2 specific activity of membrane fraction from control and EtOH-treated mouse brain increased with increasing concentrations of Ca^{2+}. The activation was observed at lower concentrations of Ca^{2+} with the maximum activation at 0.5 mM Ca^{2+}; a significant increase was observed above this concentration only with EtOH. These results suggest that EtOH may activate more than one PLA_2, requiring both lower and higher Ca^{2+} concentrations for complete activation. In nerve cells, the concentration of cytosolic free Ca^{2+} is maintained at 0.1 to 0.3 μM in the resting state and increases up to 1–2 μM during excitation [46]. A similar type of PLA_2 was reported in rat brain [44], which has been shown to be affected by increasing concentrations of Ca^{2+} [44]. Ca^{2+}-dependent association of PLA_2 with the membranes has been shown in the macrophage cell line RAW 264.7 [47], and in human

brain and rat brain synaptosomal membranes [44]. The purified rat brain PLA_2 has been found to be associated with the membrane in a Ca^{2+}-dependent manner. In the absence of Ca^{2+}, the enzyme does not bind to synaptosomal membranes [44]. $cPLA_2$ stimulated by Ca^{2+} ionophore or IgE/antigen has been shown to translocate from cytosol to the nuclear membrane in mast cells [25]. The Ca^{2+}-sensitivity of PLA_2 observed in the present study suggests that EtOH may modulate PLA_2 activity through calcium ions in the nervous system.

Among the various membrane fractions examined, the highest PLA_2 specific activity was associated with the synaptic membrane followed by the nuclear membrane. It is not clear at this time why PLA_2 specific activity in purified synaptic membrane is decreased compared with the P100 fraction. One possible explanation for the observed loss of PLA_2 specific activity in the purified fractions may be due to loss of cofactors or dissociation of loosely bound PLA_2 itself from the membrane. Treatment of membranes with 1 M KCl led to total dissociation of PLA_2 activity from membrane, indicating that the enzyme exists in a membrane-associated form rather than as an integral membrane protein. A similar type of PLA_2 has been reported in platelet lysate [48], rat kidneys [49], gerbil brain [50], and human brain [45]. These results further support the notion that EtOH may translocate the PLA_2 to the synaptic membrane by an as of yet undetermined mechanism in chronic EtOH-exposed mouse brain, thereby altering the fatty acyl composition as a part of an adaptation mechanism. This may further lead to alteration of surface receptors and second messenger systems.

PLA_2 specific activity of membrane fractions from control and EtOH-treated mouse brain was enhanced greatly in the presence of glycerol. The hydrolysis of phospholipids by $cPLA_2$ is reported to be activated by glycerol [34–36]. Further, the PLA_2 specific activity in the control and EtOH-treated membranes was inhibited by the $cPLA_2$ specific inhibitor MAFP with an IC_{50} value of 3.33 μM. This compound is an irreversible inhibitor of $cPLA_2$ and has no effect on $sPLA_2$ [36]. MAFP has been used previously to identify the role of $cPLA_2$ in signal transduction [51]. In addition, most characteristically, the PLA_2 specific activity of membranes from both control and EtOH-treated mouse brain was DTT insensitive, which is also in keeping with the characteristics of $cPLA_2$. Taken together, these results suggest that EtOH-activated PLA_2 is membrane associated and most likely has a cytosolic origin. The Ca^{2+} sensitivity (both lower and higher) of PLA_2, arachidonoyl specificity, and association of higher activity with synaptic membranes observed in the present study seem to suggest that EtOH may modulate arachidonic acid specific PLA_2 as part of an adaptation mechanism to chronic EtOH. Thus, the activation of PLA_2 in EtOH-exposed mouse brain may lead to either altered physical properties of the membrane or altered membrane function through the release of arachidonic acid and its metabolites, which may play a role in the adaptation mechanism to chronic EtOH exposure. Further studies with $cPLA_2$ antibodies and different phospholipid substrates will reveal the precise role played by PLA_2 and the role played by released arachidonic acid and its metabolites in EtOH tolerance and dependence.

The authors would like to thank Dr. Amiram I. Barkai for helpful discussion. This study was supported, in part, by a grant from NIH (AA07525).

References

1. Iqbal Z and Sze PY, Ethanol modulates calmodulin-dependent Ca^{2+}-activated ATPase in synaptic plasma membranes. *Neurochem Res* **19:** 475–482, 1994.
2. Littleton J, Little H and Laverty R, Role of neuronal calcium channels in ethanol dependence: From cell cultures to the intact animal. *Ann NY Acad Sci* **654:** 324–334, 1992.
3. Weight FF, Cellular and molecular physiology of alcohol actions in the nervous system. *Int Rev Neurobiol* **33:** 289–348, 1992.
4. Charness ME, Simon RP and Greenberg DA, Ethanol and the nervous system. *N Engl J Med* **321:** 442–454, 1989.
5. Coe IR, Yao L, Diamond I and Gordon AS, The role of protein kinase C in cellular tolerance to ethanol. *J Biol Chem* **271:** 29468–29472, 1996.
6. Snell LD, Nunley KR, Lickteig RL, Browning MD, Tabakoff B and Hoffman PL, Regional and subunit specific changes in NMDA receptor mRNA and immunoreactivity in mouse brain following chronic ethanol ingestion. *Brain Res Mol Brain Res* **40:** 71–78, 1996.
7. Alling C, Diamond I, Leslie SW, Sun GY and Wood WG, *Alcohol, Cell Membranes, and Signal Transduction in Brain.* Plenum Press, New York, 1993.
8. Macdonald RL, Ethanol, γ-aminobutyrate type A receptors, and protein kinase C phosphorylation. *Proc Natl Acad Sci USA* **92:** 3633–3635, 1995.
9. Dohrman DP, Diamond F and Gordon AS, Ethanol causes translocation of cAMP-dependent protein kinase catalytic subunit to the nucleus. *Proc Natl Acad Sci USA* **93:** 10217–10221, 1996.
10. Hunt WA, *Alcohol and Biological Membranes.* The Guilford Press, New York, 1985.
11. Sun GY and Sun AY, Effect of chronic ethanol administration on phospholipid acyl groups of synaptic plasma membrane fractions from guinea pig brain. *Res Commun Chem Pathol Pharmacol* **24:** 405–408, 1979.
12. Leslie CC, Properties and regulation of cytosolic phospholipase A_2. *J Biol Chem* **272:** 16709–16712, 1997.
13. Dennis EA, Ackermann EJ, Deems RA and Reynolds LJ, Multiple forms of phospholipase A_2 in macrophages capable of arachidonic acid release for eicosanoid biosynthesis. *Adv Prostaglandin Thromboxane Leukot Res* **23:** 75–80, 1995.
14. Clark JD, Lin LL, Kriz RW, Ramesha CS, Lin AY, Milona N and Knopf JL, A novel arachidonic acid-selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP. *Cell* **65:** 1043–1051, 1991.
15. Lehman JJ, Brown KA, Ramanadham S, Turk J and Gross RW, Arachidonic acid release from aortic smooth muscle cells induced by [Arg^8]vasopressin is largely mediated by calcium-independent phospholipase A_2. *J Biol Chem* **268:** 20713–20716, 1993.
16. Elmer GI and George FR, The role of specific eicosanoids in mediating the acute effect of narcotic effects of ethanol. *J Pharmacol Exp Ther* **277:** 308–315, 1996.
17. John GR, Littleton JM and Nhamburo PJ, Increased activity of Ca^{2+}-dependent enzymes of membrane lipid metabolism in

synaptosomal preparations from ethanol-dependent rats. *J Neurochem* **44:** 1235–1241, 1985.
18. Hungund BL, Zheng Z, Lin L and Barkai AL, Ganglioside GM1 reduces ethanol induced phospholipase A_2 activity in synaptosomal preparations from mice. *Neurochem Int* **25:** 321–325, 1994.
19. Stubbs CD, Williams BH, Pryor CL and Rubin E, Ethanol-induced modifications to membrane lipid structure: Effect on phospholipase A_2-membrane interactions. *Arch Biochem Biophys* **262:** 560–573, 1988.
20. Natsuki R, Effect of chronic ethanol on phospholipase A_2 and C activity in chick embryo brain, heart and liver. *Jpn J Alcohol Stud Drug Depend* **30:** 348–357, 1995.
21. Diez EJ, Balsinde M and Aracil SA, Ethanol induces release of arachidonic acid but not synthesis of eicosanoids in mouse peritoneal macrophages. *Biochim Biophys Acta* **921:** 82–89, 1987.
22. Goldstein DB, An animal model for testing effects of drugs on alcohol withdrawal reactions. *J Pharmacol Exp Ther* **183:** 14–22, 1972.
23. Hungund BL, Goldstein DB, Villegas F and Cooper TB, Formation of fatty acid ethyl esters during chronic ethanol treatment in mice. *Biochem Pharmacol* **37:** 3001–3004, 1988.
24. Lundquist F, The determination of ethyl alcohol in blood and tissue. *Methods Biochem Anal* **7:** 217–251, 1959.
25. Glover S, de Carvalho MS, Bayburt T, Jonas M, Chi E, Leslie CC and Gelb MH, Translocation of the 85-kDa phospholipase A_2 from cytosol to the nuclear envelope in rat basophilic leukemia cells stimulated with calcium ionophore or IgE/antigen. *J Biol Chem* **270:** 15359–15367, 1995.
26. Huttner WB, Schiebler W, Greengard P and De Camilli P, Synapsin I (protein I), a nerve terminal-specific phosphoprotein. III. Its association with synaptic vesicles studied in a highly purified synaptic vesicle preparation. *J Cell Biol* **96:** 1374–1388, 1983.
27. Jones DH and Matus AI, Isolation of synaptic plasma membrane from brain by combined flotation-sedimentation density gradient centrifugation. *Biochim Biophys Acta* **356:** 276–287, 1974.
28. Johnson MK, The intracellular distribution of glycolytic and other enzymes in rat-brain homogenates and mitochondrial preparations. *Biochem J* **77:** 610–618, 1960.
29. Abdel-Latif AA, Smith JP and Ellington EP, Subcellular distribution of sodium-potassium adenosine triphosphate acetylcholine and acetylcholinesterase in developing rat brain. *Brain Res* **18:** 441–450, 1970.
30. Ulevitch RJ, Sano M, Watanabe Y, Lister MD, Deems RA and Dennis EA, Solubilization, purification, and characterization of a membrane-bound phospholipase A_2 from the P388D1 macrophage-like cell line. *J Biol Chem* **263:** 3079–3085, 1988.
31. Dole VP, A relation between non-esterified fatty acids in plasma and the metabolism of glucose. *J Clin Invest* **35:** 150–154, 1956.
32. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
33. Raaflaub J, Application of metal buffers and metal indicators in biochemistry. *Methods Biochem Anal* **27:** 301–325, 1960.
34. Clark JD, Milona N and Knopf JL, Purification of a 110-kilodalton cytosolic phospholipase A_2 from the human monocytic cell line U937. *Proc Natl Acad Sci USA* **87:** 7708–7712, 1990.
35. Reynolds LJ, Hughes LL, Louis AI, Kramer RM and Dennis EA, Metal ion and salt effects on the phospholipase A_2, lysophospholipase, and transacylase activities of human cytosolic phospholipase A_2. *Biochim Biophys Acta* **1167:** 272–280, 1993.
36. Sharp JD, Pickard RT, Chiou XG, Manetta JV, Kovacevic S, Miller JR, Varshavsky AD, Roberts EF, Strifler BA, Brems DN and Kramer RM, Serine 228 is essential for catalytic activities of 85-kDa cytosolic phospholipase A_2. *J Biol Chem* **269:** 23250–23254, 1994.
37. Huang Z, Payette P, Abdullah K, Cromlish WA and Kennedy BP, Functional identification of the active-site nucleophile of the human 85-kDa cytosolic phospholipase A_2. *Biochemistry* **35:** 3712–3721, 1996.
38. Vadas P, Stefanski E and Pruzanski W, Characterization of extracellular phospholipase A_2 in rheumatoid synovial fluid. *Life Sci* **36:** 579–587, 1985.
39. Katsura M, Ohkuma S, Chen DZ and Kuriyama K, Ethanol-induced alteration in activities of cerebral phosphatidylinositol 4,5-biphosphate-specific and cytosolic phospholipase C in the brain: Analysis using NG 108-15 cells and brains from ethanol-inhaled mice. *Neurochem Int* **24:** 541–547, 1994.
40. Leslie CC, Voelker DR, Channon JY, Wall MM and Zelarney PT, Properties and purification of an arachidonyl-hydrolyzing phospholipase A_2 from a macrophage cell line, RAW 264.7. *Biochim Biophys Acta* **963:** 476–492, 1988.
41. Wijkander J and Sundler RA, Phospholipase A_2 hydrolyzing arachidonyl-phospholipids in mouse peritoneal macrophages. *FEBS Lett* **244:** 51–56, 1989.
42. Wijkander J and Sundler R, An 100-kDa arachidonate-mobilizing phospholipase A_2 in mouse spleen and the macrophage cell line J774. Purification, substrate interaction and phosphorylation by protein kinase C. *Eur J Biochem* **202:** 873–880, 1991.
43. Fujimori Y, Murakami M, Kim DK, Hara S, Takayama K, Kudo I and Inoue K, Immunochemical detection of arachidonyl-preferential phospholipase A_2. *J Biochem (Tokyo)* **111:** 54–60, 1992.
44. Yoshihara Y and Watanabe Y, Translocation of phospholipase A_2 from cytosol to membranes in rat brain induced by calcium ions. *Biochem Biophys Res Commun* **170:** 484–490, 1990.
45. Ross BM, Kim DK, Bonventre JV and Kish SJ, Characterization of a novel phospholipase A_2 activity in human brain. *J Neurochem* **64:** 2213–2221, 1995.
46. Balow RM, Tomkinson B, Ragnarsson U and Zetterqvist O, Purification, substrate specificity, and classification of tripeptidyl peptidase II. *J Biol Chem* **261:** 2409–2417, 1986.
47. Channon JY and Leslie CC, A calcium-dependent mechanism for associating a soluble arachidonyl-hydrolyzing phospholipase A_2 with membrane in the macrophage cell line RAW 264.7. *J Biol Chem* **265:** 5409–5413, 1990.
48. Aarsman AJ, Leunissen-Bijvelt J, Van den Koedijk CD, Neys FW, Verkleij AJ and Van den Bosch H, Phospholipase A_2 activity in platelets. Immuno-purification and localization of the enzyme in rat platelets. *J Lipid Mediat* **1:** 49–61, 1989.
49. Nakamura H, Nemenoff RA, Gronich JH and Bonventre JV, Subcellular characteristics of phospholipase A_2 activity in the rat kidney. Enhanced cytosolic, mitochondrial, and microsomal phospholipase A_2 enzymatic activity after renal ischemia and reperfusion. *J Clin Invest* **87:** 1810–1818, 1991.
50. Rordorf G, Vemura Y and Bonventre JV, Characterization of phospholipase A_2 (PLA_2) activity in gerbil brain: Enhanced activities of cytosolic, mitochondrial and microsomal forms after ischemia and reperfusion. *J Neurosci* **11:** 1829–1836, 1991.
51. Balsinde J and Dennis EA, The incorporation of arachidonic acid into triacylglycerol in 388D1 macrophage-like cells. *Eur J Biochem* **235:** 480–485, 1996.