Characterization of Phospholipase A₂ (PLA₂) Activity in Gerbil Brain: Enhanced Activities of Cytosolic, Mitochondrial, and Microsomal Forms after Ischemia and Reperfusion

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Brain phospholipase A₂ (PLA₂) activity has not been well characterized. Given the importance of this enzymatic activity for a variety of cellular functions in the brain, we characterized the subcellular distribution of PLA₂ activity in gerbil brain and evaluated how PLA₂ activity was altered by ischemia and reperfusion. Cytosolic, mitochondrial, and microsomal fractions were prepared by differential centrifugation of forebrain homogenates. PLA₂ activities of each fraction were assayed by measuring release of arachidonic acid (AA) from exogenous ¹⁴C-AA-phosphatidylcholine (PC), -phosphatidylethanolamine (PE), and -phosphatidylinositol (PI). Two forms of PLA₂ were present in the cytosolic fraction: a high-molecular-weight form, active against PC and PE, and a smaller form with an *M_r* of approximately 14 kDa, active against PE. In the mitochondrial and microsomal fractions, a single form (*M_r* ≈ 14 kDa) was dominant, active against both PC and PE. The role of PLA₂ activation in ischemic brain injury remains controversial. PLA₂ enzymatic activity was characterized in gerbil brain after 10 min of common carotid occlusion, followed by 10 min of reperfusion. Ischemic/reperfused brains had significantly higher PLA₂ specific activities in each subcellular fraction. Ischemia and reperfusion did not change the gel-filtration elution patterns of PLA₂ activity of the various forms of the enzyme. Cytosolic, mitochondrial, and microsomal activities were optimal at a pH of approximately 8.5. Cytosolic PLA₂ activity was enhanced when Ca²⁺ concentration ([Ca²⁺]) was increased over the physiological range (10⁻⁷ to 10⁻⁶ M). Mitochondrial and microsomal PLA₂ activities were also [Ca²⁺] dependent. In conclusion, gerbil brain has various forms of Ca²⁺-dependent PLA₂ activities. Ischemia and reperfusion result in stable activation of both soluble and membrane-associated forms. This stable activation of PLA₂ may play a major role in cellular injury associated with ischemia and reperfusion in the brain.

Phospholipase A₂ (PLA₂) activation in the brain results in increased tissue levels of arachidonic acid (AA). AA and its metabolic products play important roles in various aspects of neuronal function. They can act as messengers themselves, interact with other cellular messenger systems (Piomelli et al., 1987), and modulate ion channel activity (Kim and Clapham, 1989; Ordway et al., 1989; Schweitzer et al., 1990), ligand binding and uptake (Barbour et al., 1989; Saltarelli et al., 1990), and neurotransmitter release (Ohmichi et al., 1989).

The role of PLA₂ in brain cell injury associated with ischemia was suggested by the finding that brain free fatty acids (FFAs) increase rapidly during ischemia (Bazan, 1970, 1971; Galli and Spagnuolo, 1976; Yoshida et al., 1980; Ikeda et al., 1986; Abe et al., 1987), with AA showing the largest relative increase (Bazan, 1970; Yasuda et al., 1985). There is agreement that these FFAs are derived from membrane phospholipids through the activation of endogenous lipases; however, the identity of the responsible lipases remains controversial. AA can be acted upon by cyclooxygenase and lipoxygenases to generate prostaglandins, leukotrienes, and thromboxanes, which are increased in the brain after ischemia and reperfusion (Gaudet and Levine, 1979; Shohami et al., 1982; Moskowitz et al., 1984; Dempsey et al., 1986).

PLA₂ can act upon membrane phospholipids to alter membrane structure and release FFAs and lysophospholipids. The direct effects of PLA₂ on the cell membrane will change membrane permeability characteristics to Ca²⁺, alter mitochondrial function, and increase the sensitivity of cellular organelles to reactive oxygen metabolites (Farber et al., 1981; Malis and Bonventre, 1986; Bonventre et al., 1988). The elevated cellular levels of FFAs (Chan and Higgins, 1978) and lysophospholipids (Corr et al., 1984), resulting from PLA₂-induced phospholipid hydrolysis, also can be toxic to cells. AA metabolites may contribute to the vasospasm associated with ischemia/reperfusion (Moncada and Vane, 1979). Furthermore, these inflammatory intermediates may contribute to the influx of neutrophils and monocytes, which can then release more vasoactive substances and generate reactive oxygen species (Watson and Ginsberg, 1989).

Measurement of FFA or AA metabolite accumulation, however, is not a reliable measure of PLA₂ activity because other enzymes can release AA (Bonventre and Nemenoff, 1991) and the activity of reacylation enzymes may contribute in an important way to the steady-state levels of FFAs. Foudin et al. (1982) found that ischemia resulted in an inhibition of ATP-dependent incorporation of AA into phospholipids. Furthermore, AA can also be released by the combined action of phos-

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Figure 1. Fractionation of forebrain cytosolic extracts by fast protein liquid chromatography (FPLC) using a Superose 12 gel-filtration column. Extracts were applied to a 24-ml column and eluted at 0.5 ml/min. 1-ml fractions were collected. PLA₂ activity of each fraction was measured using PC (A) and PE (B). The column was calibrated using the following standards (arrows): bovine serum albumin (M_r , 66 kDa), ovalbumin (45 kDa), porcine pancreatic PLA₂ (13.5 kDa), and vitamin B₁₂ (1.35 kDa). One peak of activity was observed with PC, whereas two peaks were seen when PE was used as substrate. In C, the protein concentration of each fraction is presented.

pholipase C followed by diacylglyceride lipase (Avelano and Bazan, 1975) or by phospholipase A₁ followed by lysophospholipase. Therefore, it is inappropriate to equate elevated steady-state levels of FFAs or AA metabolites with activation of PLA₂.

In only a few studies has PLA₂ activity been measured directly (Edgar et al., 1982), and even when found to be enhanced, increased PLA₂ activity has been reported to be transient (1–5 min), followed by a rapid return toward normal control levels. Thus, investigators (Yasuda et al., 1985; Abe et al., 1987) studying cerebral ischemia have concluded that increased tissue levels of AA are likely to come primarily from phosphatidylinositol (PI) via PI-specific phospholipase C with subsequent release of

AA by diacylglyceride lipase. A similar controversy exists with regard to PLA₂ activity following experimental head injury. The increase of measured FFAs has been attributed by some investigators to the activation of PLA₂ (Shohami et al., 1989), and by others to the activation of phospholipase C followed by diacylglyceride lipase (Wei et al., 1982). In order to understand the events triggering cell and tissue injury, it is important to define the characteristics and regulation of PLA₂ and the role played by Ca²⁺ and other modulators in PLA₂ activation with ischemia.

In the present study, we partially characterized the brain cytosolic, mitochondrial, and microsomal PLA₂ activity and measured PLA₂ activity directly in each cellular fraction prior and subsequent to cerebral ischemia with reperfusion. Our data indicate the presence of multiple forms of PLA₂ activity in the brain with Ca²⁺ sensitivities that are consistent with important physiological and pathophysiological roles in each fraction. The data also demonstrate that cytosolic, mitochondrial, and microsomal PLA₂ activities are stably enhanced with ischemia and reflow.

Materials and Methods

Materials. Radioactively labeled phospholipid substrates, 1-stearoyl-2-(1-¹⁴C) arachidonyl phosphatidylcholine (PC) and 1-acyl-2-(1-¹⁴C) arachidonyl L-3-phosphatidyl ethanolamine (PE) were purchased from Amersham Corp.; 1-stearoyl-2-(1-¹⁴C) arachidonyl phosphatidylinositol (PI) and 1-hexadecyl-2-(³H)-arachidonyl phosphatidylcholine (1-hexadecyl-2-arachidonyl-PC) were obtained from New England Nuclear Comp. Arachidonic acid was obtained from Sigma Chemical Co. Silica-gel LK5D plates were obtained from Whatman, and scintillation fluid (Ecoscint®) was from National Diagnostic. Gel-filtration chromatography standards were purchased from Sigma.

Animal preparation and brain sampling technique. Male Mongolian gerbils, weighing 50–70 gm (Tumblebrook Farms, West Brookfield, MA), were allowed free access to food and water. Transient forebrain ischemia was produced by occluding both common carotid arteries, as previously described (Uemura et al., 1990). Animals were lightly anesthetized with ethyl ether (E. Merck Science). Body temperature was maintained at 36–37°C during vascular occlusion by using a homeothermal blanket control unit (Harvard Apparatus). The common carotid arteries were exposed by a ventral midline incision and gently separated from the carotid sheath by blunt dissection. Both carotid arteries were occluded with a small aneurysmal clip, and the vessels were examined beyond the clips for absence of flow. After 10 min, the clips were removed, and restoration of carotid blood flow was verified by direct observation. The animals were then decapitated after 10 min of reperfusion, and the forebrain was quickly removed. Sham-operated animals were treated in an identical fashion with the exception of carotid occlusion.

Preparation of cellular fractions. Forebrains were quickly transferred into 5 ml of buffer containing 250 mM sucrose, 50 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, and the protease inhibitors pepstatin (20 μM), leupeptin (20 μM), trasylol (1000 kallikrein-inactivating units/ml), and phenylmethylsulfonyl chloride (0.1 mM) at 4°C and pH 7.5, then homogenized with a Polytron® homogenizer (Brinkman).

The homogenate was centrifuged at 900 × *g* for 10 min. The pellet was discarded, and the supernatant was centrifuged at 9000 × *g* for 20 min. The pellet was designated as the crude mitochondrial fraction containing primarily mitochondria and synaptosomes (Woelk and Porcellati, 1973). The supernatant was centrifuged at 105,000 × *g* for 60 min. The resultant supernatant was taken to be the cytosolic fraction, and the pellet, the microsomal fraction (Gray and Strickland, 1982). The mitochondrial and microsomal pellets were resuspended and passed through a 22-gauge needle 10 times and incubated in a buffer containing 50 mM Tris HCl, 1 mM EGTA, 1 mM EDTA, and 1 M KCl (pH, 7.4) for 1 hr at 4°C. The suspension was then centrifuged at 12,000 × *g* for 30 min, and PLA₂ activity of the supernatant was determined. The recovery of mitochondrial and microsomal PLA₂ activity in the supernatant was greater than 90% of the activity measured in the initial pellet suspension. Protein content of each fraction was determined by the Bio-Rad reaction, and samples were matched for protein concentration prior to the PLA₂ assay.

Phospholipase A_2 assay. PLA₂ activity of each cellular fraction was measured as previously described with modifications (Gronich et al., 1988); 1-stearoyl-2-(1-¹⁴C) arachidonyl-L-3-phosphatidylcholine (PC), 1-acyl-2-(1-¹⁴C) arachidonyl-phosphatidylethanolamine (PE), 1-stearoyl-2-(1-¹⁴C) arachidonyl-phosphatidylinositol (PI), and 1-hexadecyl-2-arachidonyl-phosphatidylcholine were used as substrates. The substrates were dried down under N₂ and resuspended in dimethylsulfoxide. The final assay substrate concentration was 15 μ M. The assay was initiated by adding 34 μ l of sample with or without 2.5 mM Ca²⁺ (0.5 mM in excess of the concentration of EDTA and EGTA) at the physiological pH of 7.5. The reaction was carried out for 30 min at 37°C and stopped with a quench solution containing 2% acetic acid in ethanol with 100 μ g/ml free arachidonic acid; 50 μ l of the mixture was then spotted onto heat-activated silica-gel thin-layer chromatography plates and developed with the organic phase of ethyl acetate, isooctane, H₂O, and acetic acid (55:75:100:8). Blank samples were run containing extract buffer. Lipids were visualized by iodine staining.

The phospholipid and free arachidonic acid bands were scraped into scintillation vials; 3 ml of Ecoscint scintillation fluid was added, and radioactivity was either counted in a Hewlett-Packard scintillation counter. Alternatively, radioactivity was evaluated directly on the thin-layer chromatography plates using a Berthold Automatic TLC-Linear Analyzer (Berthold, Germany). Results are expressed as picomoles of arachidonic acid released per min per mg of protein.

Chromatography. Samples (300–500 μ l) were loaded on a 24-ml Superose 12 gel-filtration column (Pharmacia LKB Biotechnology Inc.), equilibrated in 50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, and 1 mM EDTA, and were eluted with the same buffer at 0.5 ml/min. The column was calibrated using the following standards: bovine serum albumin (66 kDa) ovalbumin (45 kDa), porcine phospholipase A₂ (13.5 kDa), and vitamin B₁₂ (1.35 kDa). One-milliliter fractions were collected and assayed for PLA₂ activity and protein concentration using the PLA₂ and protein assays described above.

pH dependency of PLA₂ activity. Samples of the cytosolic, crude mitochondrial, and microsomal fractions were diluted with buffers of different pH (pH 5.0–11.0), and actual pH and PLA₂ activity of each sample were determined in the presence of Ca²⁺. Tris-HCl buffer was employed as a buffer to make up solutions at pH 7.5 and below, and glycine/NaOH buffer was used for pH 8.0 and above. All the samples were matched for protein.

Ca²⁺ dependency of PLA₂ activity. Extracts were diluted 1:8 with buffers consisting of NaCl, 140 mM; HEPES, 25 mM; and varying amounts of CaCl₂ with or without 1 mM EGTA (pH, 7.5). Enzymatic activity was determined after an aliquot of the mixture was taken to measure the free Ca²⁺ concentration. Enzymatic activity and free calcium concentration were measured at 37°C. Ca²⁺ concentration was determined using the dual-wavelength fluorescence characteristics of fura-2 free acid, for Ca²⁺ concentrations below 1 μ M, using a dual-wavelength spectrofluorometer (Deltascan Spectrofluorometer, Photon Technology Inc., Princeton, NJ). For concentrations greater than 1 μ M, the free Ca²⁺ concentration was determined with a Ca²⁺-selective electrode, which we constructed and calibrated as previously described (Bonventre et al., 1986).

Statistics. Data are presented as means $\pm$ 1 standard error of the mean. The Student's *t* test was used to evaluate statistical significance. A *p* value of <0.05 was considered statistically significant. Each experiment was repeated at least five times.

Results

Chromatography of cytosolic, mitochondrial, and microsomal extracts

To partially characterize brain PLA₂, cytosolic, mitochondrial, and microsomal extracts were separated by Superose 12 gel-filtration chromatography. In the cytosolic extract, when PC was used as substrate, there was one peak of PLA₂ activity migrating with an approximate molecular weight of 60 kDa (Fig. 1A). When PE was used as substrate, in addition to this larger form there was a second peak of activity with a smaller molecular weight (*M_r* $\approx$ 14 kDa; Fig. 1B). In mitochondrial (Fig. 2A,B) and microsomal (Fig. 3A,B) extracts, there was a single peak of PLA₂ activity with an estimated molecular weight of approximately 14 kDa when either PC or PE was used as substrate.

Figure 2. Fractionation of forebrain mitochondrial extracts by FPLC Superose 12 gel-filtration chromatography. The crude mitochondrial extract was applied to a 24-ml Superose 12 gel-filtration column, and PLA₂ activity was measured using PC (A) or PE (B) as substrate. One peak of activity was seen with either substrate. The protein profile in the fractions is presented in C. Column calibration standards (arrows) are described in Figure 1.

Phospholipase A_2 activity after ischemia and reperfusion

The PLA₂ activities of cytosolic, mitochondrial, and microsomal extracts were measured after 10 min of ischemia and 10 min of reperfusion and compared with the activities of control brain extracts. As demonstrated in Figure 4, there was a significant increase of PLA₂ specific (Fig. 4A,B) and total (Fig. 4C,D) activities in each extract when PC or PE was used as substrate. Enhancement of cytosolic PLA₂ activity after ischemia and reperfusion was seen when PC, PE, PI, or 1-hexadecyl-2-arachidonyl-PC was used as substrate (Fig. 5). The highest specific activity was found when 1-hexadecyl-2-arachidonyl-PC was used as substrate for ischemic cytosolic extracts.

To further characterize brain PLA₂ after ischemia and reperfusion, cytosolic, mitochondrial, and microsomal extracts of ischemic brain were fractionated by Superose 12 gel-filtration

Figure 3. Fractionation of forebrain microsomal extracts by FPLC Superose 12 gel-filtration chromatography. Fractionation conditions were identical to those used for cytosolic and mitochondrial extracts. PLA₂ activity was measured using PC (*A*) or PE (*B*) as substrate. One peak of activity was seen with either substrate. The protein profile in the fractions is presented in *C*. Column calibration standards (arrows) are described in Figure 1.

chromatography. PLA₂ activity of the cytosolic ischemic extract migrated as a single peak of activity eluting at fraction 13 when PC was used as substrate and as two peaks eluting at fractions 13 and 17 when PE was substrate. With both substrates, the migration pattern was identical to that seen in preischemic control extracts (Fig. 1).

When PC or PE was used as the substrate, mitochondrial and microsomal ischemic extracts displayed a single peak of activity eluting at fraction 17 at the same position as the peak of activity in control mitochondrial (Fig. 2) and microsomal (Fig. 3) samples.

pH dependency of cytosolic, mitochondrial, and microsomal forms of brain PLA₂

Samples of cytosolic, mitochondrial, and microsomal extracts were diluted with buffers of different pH (pH, 5.0–11.0), and

PLA₂ activity was determined as a function of measured pH (Fig. 6). Each extract had an alkaline pH optimum (≈ 8.5). At each alkaline pH, cytosolic, mitochondrial, and microsomal extracts from ischemic and reperfused brains had greater PLA₂ activity than extracts from control brains (shown for a cytosolic extract in Fig. 6*A*).

Calcium dependency of cytosolic, mitochondrial, and microsomal forms of brain PLA₂

PLA₂ activity in each of the cellular fractions is $[Ca^{2+}]$ dependent. As shown in Figure 7, with PE as substrate, the activity of cytosolic, mitochondrial, and microsomal extracts are markedly enhanced in the presence of 50 μM Ca^{2+} . This Ca^{2+} dependency of cytosolic, mitochondrial, and microsomal fractions was unaffected by ischemia and reperfusion (data not shown). The specific Ca^{2+} concentration dependency of cytosolic PLA₂ activity is shown in Figure 8. Note that cytosolic PLA₂ activity was enhanced when Ca^{2+} concentration was increased greater than 100 nM.

When cytosolic, mitochondrial, and microsomal PLA₂ activities were assayed after fractionation by Superose 12 gel filtration in the presence or absence of Ca^{2+} , no peak of activity was found in the absence of Ca^{2+} .

Discussion

Two general types of PLA₂ have been recognized: secretory and intracellular forms (Waite, 1985). The extensively studied secretory forms of the enzyme, found in insect venom, snake venom, and pancreatic extracts, are small, soluble proteins with molecular weights of 12–20 kDa. Much less is known about the structure, function, or regulation of the intracellular forms of PLA₂, especially brain PLA₂s.

Our results reveal the presence of two distinct forms of PLA₂ in the cytosolic fraction of the brain. The large-molecular-weight form migrates on Superose 12 in a pattern similar in size to the form described in the human brain (Cooper and Webster, 1972). However, this previously described form was not Ca^{2+} dependent and had an acid pH optimum; it therefore is distinct from the cytosolic form we found. The form we found is similar to that recently purified from rat kidney, whose molecular mass by polyacrylamide electrophoresis was found to be approximately 110 kDa (Gronich et al., 1990). In addition to this form, we found a smaller cytosolic form active against PE but not PC. It is unlikely that this smaller form is a contaminant from the mitochondria or microsomes because the smaller-molecular-weight forms extracted from mitochondria and microsomes have activity against both PC and PE.

In both crude mitochondrial and microsomal subcellular fractions from control brain, there were single peaks of activity against PC and PE with apparent molecular weight of approximately 14 kDa when gel-filtration chromatography was performed. This size, the alkaline pH optimum, and the Ca^{2+} dependency are all characteristics similar to those of previously described mitochondrial and microsomal forms from other rat tissues such as liver (Waite and Sisson, 1971; Aarsman et al., 1989) and spleen (Ono et al., 1988).

In the brain, a form of microsomal PLA₂ was described by Gray and Strickland (1982). This form was also Ca^{2+} dependent but had an optimal pH at 7.4 and a substrate specificity for PI. A Ca^{2+} -insensitive mitochondrial form was described by Woelk and Porcellati (1973) that had an optimal activity at pH 8.6.

Figure 4. Cytosolic, mitochondrial, and microsomal PLA₂ specific (*A, B*) and total (*C, D*) activities in control extracts and extracts of forebrain removed after 10 min of ischemia and 10 min of reperfusion. Cytosolic, mitochondrial, and microsomal PLA₂ specific and total activities were significantly increased in the ischemic forebrain extracts as compared to control extracts, whether PC (*A, C*) or PE (*B, D*) was used as substrate. The numbers in parenthesis represent the number of experiments. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, compared with controls. Error bars represent standard errors of the mean (SEM).

The Ca²⁺-dependent mitochondrial and microsomal PLA₂ activities that we found therefore represent forms of membrane-associated PLA₂ distinct from previously described forms in the brain. In our study, a large percentage of membrane (mitochondrial and microsomal)-associated PLA₂ activity was solubilized with 1 M KCl, indicating that the enzymes existed as membrane-associated forms rather than as integral membrane proteins.

The cytosolic PLA₂ activity was enhanced at Ca²⁺ concentrations in the physiological range observed in neurons after hormone stimulation (Miller, 1988), glutamate exposure (MacDermott et al., 1986; Murphy et al., 1987), and membrane depolarization (Miller, 1988). Mitochondrial PLA₂ activity was also Ca²⁺ dependent. The Ca²⁺-sensitivity characteristics are consistent with important physiological and pathophysiological roles for both cytosolic and mitochondrial enzymes in the intact cell (Moskowitz et al., 1982).

Phospholipid degradation likely contributes to ischemic tissue injury in brain (Bazan, 1970), kidney (Smith et al., 1980), heart (Chiarello et al., 1987; Sen et al., 1988), intestine (Otamiri et al., 1987), and liver (Chien et al., 1978). In the brain, phospholipid breakdown is also associated with a variety of other

Figure 5. Cytosolic PLA₂ activity against various substrates. Cytosolic PLA₂ specific activity was increased in postischemic forebrain extracts when PC, PE, PI, or 1-hexadecyl-2-arachidonyl-PC was used as the substrate. The highest specific activity was found in the postischemic samples when 1-hexadecyl-2-arachidonyl-PC was used as substrate. ** $p < 0.005$, *** $p < 0.001$, compared with controls. Error bars represent SEM.

Figure 6. pH dependency of cytosolic (A), mitochondrial, and microsomal (B) PLA₂ activities. PLA₂ activity was determined using PC as substrate. Cytosolic, mitochondrial, and microsomal extracts have alkaline pH optima. Ischemia does not alter the pH optima of the cytosolic PLA₂ activity.

pathophysiological processes (Bazan, 1971; Shohami et al., 1989). The demonstration of AA release and prostaglandin synthesis (Moskowitz et al., 1984; Dempsey et al., 1986) with brain ischemia is consistent with a role for PLA₂ in ischemic brain cell damage but does not prove PLA₂ involvement. Other enzymatic processes, besides PLA₂ activity, can result in elevated tissue FFA levels (Bonventre and Nemenoff, 1991). For example, Abe et al. (1987) demonstrated that AA was liberated during ischemia from inositol-containing phospholipids by phospholipase C followed by diacylglyceride lipase and monoglyceride lipase. Wei et al. (1982) also showed activation of phospholipase C after brain injury. Phospholipase A₁ followed by lysophospholipase can also increase free AA levels. Plasmalogenase and

Figure 7. Calcium dependency of cytosolic, mitochondrial, and microsomal PLA₂ activity. PLA₂ activity was determined using PE as substrate. PLA₂ activity of each fraction was assayed in the presence (50 μM) or absence (<10 nM) of Ca²⁺. *** $p < 0.001$ compared with controls. Numbers in parenthesis represent the number of experiments. Error bars represent SEM.

lysophospholipase can release AA from plasmalogens. Furthermore, inhibition of reacylation mechanisms can also enhance tissue levels of AA. Das et al. (1986) showed a decrease of lysophosphatidylcholine acetyltransferase in ischemic pig myocardium.

Rarely has PLA₂ enzymatic activity been directly determined with ischemia. It has been generally assumed that increases in cytosolic Ca²⁺ concentration brought about by ischemia will activate a Ca²⁺-dependent PLA₂ enzyme.

Direct evidence for PLA₂ activation with ischemia was found in extracts of acetone-dried brain tissue after 1 min of bilateral carotid occlusion in the gerbil (Edgar et al., 1982); however, PLA₂ activity returned to baseline levels by 5 min of ischemia, and no correlation was found between PLA₂ activity and the tissue levels of FFAs during and after ischemia. Sun and Foudin (1984) measured enhanced release of lysolecithin after brain ischemia. Enhanced PLA₂ activity was also found in homogenates of small intestinal mucosal cells after ischemia and reperfusion (Otamiri et al., 1987). Kawaguchi and Yasuda (1988) reported increases of mitochondrial and microsomal PLA₂ activity in hypoxic myocardium. This activity had a substrate specificity for phosphatidylethanolamine, but the enzymatic activity was not further characterized. By contrast, homogenate and mitochondrial PLA₂ enzymatic activity was decreased 20 min after coronary artery ligation in the ischemic area of the isolated rat heart (Bentham et al., 1987), and this led to a recent conclusion that PLA₂ is not important in the etiology of ischemic injury (Van der Vusse et al., 1989).

Our data show that ischemia followed by reperfusion enhances cytosolic, mitochondrial, and microsomal PLA₂ activities. Increased mitochondrial PLA₂ activity may have serious consequences for tissue injury secondary to ischemia. The phospholipids of mitochondrial membranes are enriched with unsaturated fatty acids (Okayasu et al., 1985) and therefore are particularly good substrates for PLA₂ action. Activation of mi-

Figure 8. Calcium dependency of cytosolic PLA₂ activity. Activity was determined using PC as substrate. Cytosolic PLA₂ activity increased when [Ca²⁺] was increased over the physiological range (0.1–1.0 μ M).

tochondrial PLA₂ results in hydrolysis of cardiolipin (diphosphatidylglycerol), which is a critical component of the catalytic subunits of the electron transport chain (Fry and Green, 1981), ATP synthetase (Santiago et al., 1973; Agarwal and Kalra, 1983), and adenine nucleotide translocase (Beyer and Klingenberg, 1985). Inability to reverse mitochondrial dysfunction upon reperfusion correlates with inability to reverse ischemic cell injury (Trump et al., 1989).

While elevated cytosolic Ca²⁺ concentration may be important for activation of the PLA₂ enzymes *in vivo* with ischemia, the preservation of enhanced activity *in vitro* under conditions of our assay, where [Ca²⁺] is fixed, suggests a stable modification of the enzymes. Thus, ischemia and reperfusion may result in a covalent modification of the enzyme, and PLA₂ activation may prevail even if [Ca²⁺] levels return to baseline values *in vivo* after ischemia. This may explain, for example, why AA continues to accumulate in brain tissues after reperfusion (Nakano et al., 1990).

PLA₂ may be regulated via phosphorylation by protein kinase C (PKC; Bonventre and Swidler, 1988). PKC is activated during ischemia (Onodera et al., 1989). It is possible that phosphorylation of the enzyme enhances its activity. PLA₂ could also be associated with a regulatory protein. PKC has been suggested to phosphorylate a protein that inhibits PLA₂ activity by binding to the phospholipid substrate (Touqui et al., 1986). A regulatory protein is unlikely to explain our findings, however, because we have found (G. Rordorf, Y. Uemura, and J. V. Bonventre, unpublished observations) that the PLA₂ activities in the peak chromatography fractions of mitochondrial and microsomal samples are greater in forebrains taken after ischemia and reperfusion, suggesting enhanced activity in the PLA₂ proteins themselves.

In conclusion, we have demonstrated that multiple forms of PLA₂ are present in the cytosolic and membrane (mitochondrial and microsomal) compartments of gerbil brain. These forms differ from previously described forms and from each other. The Ca²⁺ sensitivity of both cytosolic and membrane-bound PLA₂ activities indicates that the enzymes are likely regulated

by Ca²⁺ *in vivo*. After ischemia and reperfusion, cytosolic, mitochondrial, and microsomal PLA₂ enzymatic activities were enhanced. These stable modifications of enzymatic activity cannot be explained by Ca²⁺ alone and suggest that other regulatory influences may play an important role in PLA₂ activation and mediation of cellular injury after an ischemic insult.

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