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

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Abstract

Phospholipase A_2 (PLA_2) activities in cytosolic, mitochondrial, and microsomal fractions of rat kidneys were characterized under control conditions, after ischemia, and subsequent to ischemia and reperfusion. Two forms of PLA_2 activity were present in the cytosolic fraction: a high molecular weight form, active against phosphatidylcholine (PC), and phosphatidylethanolamine (PE), which upon purification has a molecular mass of 110 kD; and a smaller form ($M_r \approx 14$ kD), active against PE. In mitochondrial and microsomal fractions a single form ($M_r \approx 14$ kD), active against both PC and PE, was dominant. Activities in each fraction were optimal at pH 8.5–9.5. Cytosolic PLA_2 activity was enhanced when Ca^{2+} concentration ($[Ca^{2+}]$) was increased over the range of 10^{-7} to 10^{-6} M. Mitochondrial PLA_2 activity required higher $[Ca^{2+}]$ for activation ($> 10^{-6}$ M).

After 45 min of ischemia cytosolic PLA_2 activity was decreased, whereas mitochondrial and microsomal activities were increased. When ischemia was followed by 1 h of reperfusion, cytosolic, mitochondrial, and microsomal activities were enhanced. Ischemia alone did not change the gel filtration chromatography patterns of PLA_2 activity, but ischemia and reperfusion resulted in the appearance of a new peak of activity in cytosolic and mitochondrial fractions ($M_r \approx 2$–3 kD).

Thus, the rat kidney has multiple forms of PLA_2 activity, likely representing distinct enzymes, with Ca^{2+} dependencies suggesting regulation by Ca^{2+} in vivo. Ischemia and reperfusion result in stable increases of PLA_2 activity in each subcellular fraction, perhaps related to covalent modifications of PLA_2's, which likely account for membrane phospholipid degradation, and increased tissue levels of unsaturated free fatty acids. (*J. Clin. Invest.* 1991. 87:1810–1818.) Key words: phospholipids • acute renal failure • acyl hydrolases • calcium • fatty acids

Introduction

Phospholipase A_2 (PLA_2)[1] has been proposed to play an important role in cell injury associated with ischemia (1). This enzyme acts on membrane phospholipids at the sn-2 position to generate lysophospholipids and free fatty acids. The resultant PLA_2-induced changes in phospholipid integrity and the toxic actions of free fatty acids and lysophospholipids may be critical to the altered plasma membrane and mitochondrial permeability properties and bioenergetic capacity associated with ischemia and reperfusion. The lipid peroxidation that occurs with ischemia and reperfusion may result in enhanced susceptibility of cellular membranes to PLA_2 (2, 3). The potential importance of PLA_2 activity in ischemic cell injury is supported by the protection that PLA_2 inhibitors afford against phospholipid degradation and the associated reduction in infarct size after coronary artery occlusion in rats (4) and also the partial preservation of the mucosal permeability barrier in the ischemic and reperfused intestine, attributed to PLA_2 inhibition (5).

The mitochondria represent particularly important sites of PLA_2 action resulting in mitochondrial dysfunction which may play an important role in the pathophysiology of ischemic injury (6, 7). Changes in mitochondrial membrane integrity could markedly impair ATP generation and hence reduce the energy currency required for recovery processes (8). Our previously reported data led us to conclude that PLA_2 activation, consequent to calcium and reactive oxygen species exposure during reoxygenation, played an important role in electron transport chain damage localized to NADH coenzyme Q reductase, decreased F_1-ATPase activity, decreased ADP-ATP translocase activity, and increased mitochondrial membrane permeability to H^+ ions (3, 6). Exogenous PLA_2 treatment of hypoxic proximal tubules results in severe cellular injury and significant decreases in uncoupled respiratory rates of the tubules, reflective of electron transport chain damage (9).

Previous studies in kidney (10), brain (11), and heart (12) have implicated PLA_2 activation in the mediation of ischemia-related tissue injury on the basis of measured increases in free fatty acids in ischemic and postischemic tissue. The increases in unsaturated fatty acids are generally greater than those of saturated fatty acids, providing further evidence for the activation of PLA_2, since the sn-2 position of phospholipids is enriched with unsaturated fatty acids. Free fatty acid release, however, is not a reliable measure of PLA_2 activity since other enzymes can release fatty acids (12, 13) and activity of reacylation enzymes may contribute in an important way to the steady-state levels of free fatty acids. In only a few studies has PLA_2 activity been measured directly. In fact, in a study in the heart (14) the cytosolic PLA_2 activity was measured directly and found to be decreased, leading the authors and others (15) to question whether PLA_2 plays an important role in ischemic injury.

In the present study we partially characterized the kidney cytosolic, mitochondrial, and microsomal PLA_2 activities and measured PLA_2 activity directly in cytosolic, mitochondrial, and microsomal fractions before and after renal ischemia with

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1. *Abbreviations used in this paper:* AA, arachidonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLA_2, phospholipase A_2.

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and without reperfusion. Our data indicate the presence of multiple forms of PLA_2 activity in the kidney with calcium sensitivities that are consistent with important physiological and pathophysiological roles both in cytosolic and mitochondrial function and dysfunction. The data also demonstrate that PLA_2 activity is stably enhanced in each of the cytosolic, mitochondrial, and microsomal compartments with ischemia and reflow.

Methods

Materials. Radioactively labeled phospholipid substrates (1-acyl-2-[1-^{14}C]arachidonyl phosphatidylethanolamine [PE] and 1-acyl-2-[1-^{14}C]-arachidonyl phosphatidylcholine [PC]) were obtained from Amersham Corp., Arlington Heights, IL. Porcine pancreatic PLA_2, arachidonic acid (AA), and molecular weight standards were obtained from Sigma Chemical Co., St. Louis, MO. Silica gel LK5D plates were from Whatman, Inc., Clifton, NJ. Scintillation fluid (Ecoscint) was purchased from National Diagnostics, Inc., Somerville, NJ.

Animal preparation. Male Sprague-Dawley rats weighing 200–250 g were anesthetized with sodium pentobarbital (Pentothal, 65 mg/kg body weight) administered intraperitoneally and the animal was placed on a heated operating table. After a tracheostomy was performed, the right jugular vein was exposed and cannulated for the administration of 0.9% NaCl at 0.02 ml/min. The left kidney was exposed through a midline laparotomy incision. The left renal pedicle was isolated and occluded, for the time indicated, with a microaneurysm vascular clamp (Roboz Surgical Instrument Co., Inc., Washington, DC). The contralateral kidney served as a control. In addition, sham-operated animals were used as controls. In these sham animals the left renal pedicle was isolated but not clamped.

In some experiments both kidneys were removed immediately at the end of the ischemic period. In other animals the vascular clamp was released after 45 min of ischemia and kidneys were removed after 1 h of reperfusion. In order to control for possible effects of unilateral kidney ischemia on the contralateral kidney, control kidneys were also taken from animals not previously operated upon.

Rats used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and the Massachusetts General Hospital, and those prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

Subcellular fractionation and extraction of PLA_2. Excised kidneys were rinsed in ice-cold buffer and immediately minced with scissors. Pieces were transferred into homogenizing buffer, containing 50 mM Tris-HCl (pH 7.5), 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, and phenylmethylsulfonyl chloride (0.1 mM), aprotinin (1,000 kallikrein-inactivating units/ml), leupeptin (20 μM), and pepstatin (20 μM) as protease inhibitors. Kidneys were homogenized with a homogenizer (Polytron, Brinkmann Instruments Co., Kriens-Luzern, Switzerland) in 10 ml of buffer for 90 s. The homogenate was then successively centrifuged for 10 min at 900 *g* (to sediment cell debris and nuclear and plasma membranes), followed by 20 min at 9,000 *g* (to sediment mitochondria) and 60 min at 100,000 *g* (to obtain microsomes) at 4°C (16). The high-speed supernatant represents the cytosolic fraction.

Various solutions and detergents were tested for their ability to extract PLA_2 activity from the kidney membrane fractions: the original homogenizing buffer; 50 mM Tris buffer with and without 0.3% lithium dodecyl sulfate, 5 mM cholic acid, 1% octyl glucoside, 1 M KCl, or 1 M NaCl. An extraction buffer consisting of 50 mM Tris-HCl buffer, 1 mM EGTA, 1 mM EDTA, and 1 M KCl resulted in the most efficient extraction of PLA_2 activity from mitochondrial and microsomal membrane fractions. Therefore the pellets were resuspended in this buffer by passing them through a 2-ml syringe with a 21-gauge needle at least 15–20 times. After 1 h of incubation at 4°C the resuspended pellets were centrifuged for 60 min at 100,000 *g*. Greater than 90% of total mitochondrial or microsomal KCl-suspension activity was recovered in the supernatant fraction after centrifugation.

PLA_2 assay. PLA_2 activity was measured as previously described with some modifications (17). 1-stearoyl-2-[1-^{14}C]arachidonyl-PC, and 1-palmitoyl-2-[1-^{14}C]arachidonyl-PE were used as substrates. Protein contents were measured using a protein analysis kit (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as a standard. All samples were matched for protein before the assay. Substrates were dried down under N_2 and resuspended in dimethylsulfoxide. 2 μl of substrate (final concentration 15 μM) was pipetted into an Eppendorf microcentrifuge tube. Reactions were initiated by the addition of each sample with or without an amount of Ca^{2+} (4 mM) which was 2.5 mM greater than the additive concentrations of EGTA and EDTA, at pH 7.5. The mixture was incubated for 30 min at 37°C and the reaction was terminated by the addition of ethanol containing 2% (vol/vol) acetic acid and 100 μg/ml free AA. Release of arachidonic acid was analyzed using thin-layer chromatography. 50 μl of the reaction mixture was spotted onto heat-activated silica gel thin-layer chromatography plates and developed in the organic phase of ethyl acetate/isooctane/H_2O/acetic acid (55:75:100:8). Lipids were visualized by I_2 staining. The phospholipid and free AA bands were scraped and radioactivity was counted with a liquid scintillation counter (Hewlett-Packard Co., Palo Alto, CA) in 3 ml of Ecoscint. Specific activity of PLA_2 was expressed as picomoles of AA released from PC or PE per minute per milligram of protein at 37°C. Total activity of PLA_2 in each subcellular fraction was also determined.

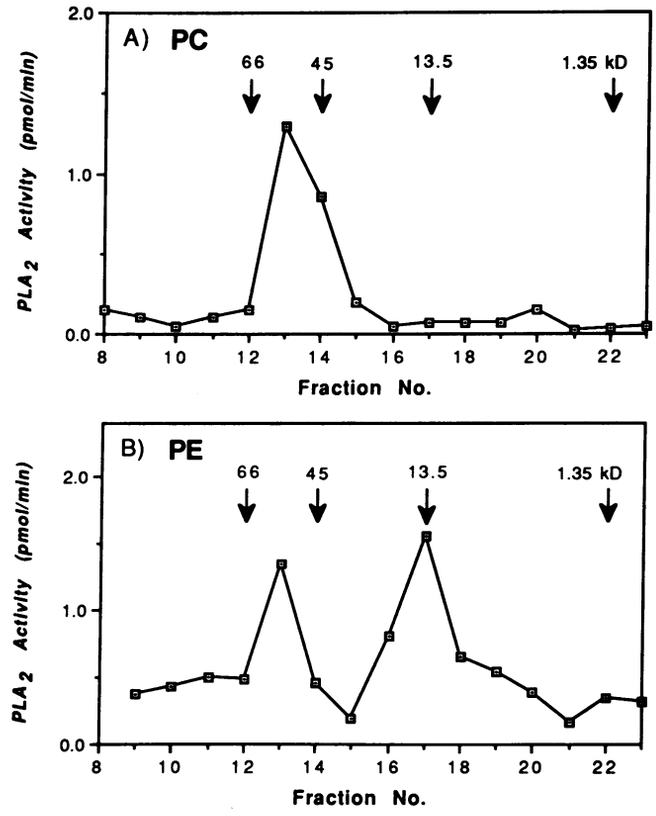

Figure 1. Fractionation of kidney cytosolic extracts by FPLC Superose 12 gel filtration. Extract was applied to a 24-ml column and 1-ml fractions were collected. PLA_2 activity of each of the fractions was measured using (*A*) PC; (*B*) PE as substrates. The column was calibrated using bovine serum albumin (M_r = 66 kD), ovalbumin (45 kD), porcine pancreatic PLA_2 (13.5 kD), and vitamin B_{12} (1.35 kD). Cytochrome *c* oxidase (12.4 kD) elutes between fractions 17 and 18 on this column.

FPLC fractionation. 250–400-μl samples of each subcellular fraction were loaded onto a 24-ml gel filtration column (Superose 12, Pharmacia LKB Biotechnology Inc., Piscataway, NJ), previously equilibrated with 50 mM Tris, 0.15 M NaCl, 1 mM EDTA, and 1 mM EGTA buffer at 4°C. Samples were run in this buffer at a flow rate of 0.5 ml/min. The column was calibrated using, as standards, blue dextran (M_r = 2,000 kD), bovine serum albumin (66 kD), ovalbumin (45 kD), porcine pancreas PLA_2 (13.5 kD), horse heart cytochrome *c* oxidase (12.4 kD), and vitamin B_{12} (1.35 kD). 1-ml fractions were collected and assayed for PLA_2 activity. The protein concentration of each fraction was monitored by absorbance at 280 nm.

PLA_2 activity pH dependency. Samples of the cytosolic and mitochondrial fractions were diluted with buffers of different pH (pH 5.0–11.0). The actual pH and PLA_2 activity of each sample were determined in the presence of Ca^{2+}. Tris-HCl buffer was employed to make up solutions of pH 7.5 or less and glycine/NaOH buffer was used for pH 8.0 and above. All the samples were matched for protein.

PLA_2 activity Ca^{2+} dependency. Extracts were diluted 1:8 with buffers consisting of NaCl 140 mM, Hepes 25 mM, and varying amounts of $CaCl_2$, with or without EGTA, 1 mM at pH 7.5. Enzymatic activity was determined after an aliquot of the mixture was taken to measure the free calcium concentration. Enzymatic activity and free calcium concentration were measured at 37°C. For Ca^{2+} concentrations below 1 μM, Ca^{2+} concentration was determined using the dual wavelength fluorescence characteristics of fura-2 free acid, using a dual wavelength spectrofluorometer (Deltascan Spectrofluorometer, Photon Technology Inc., Princeton, NJ). For concentrations > 1 μM the free Ca^{2+} concentration was determined with a Ca^{2+}-selective electrode, which we constructed and calibrated as previously described (18).

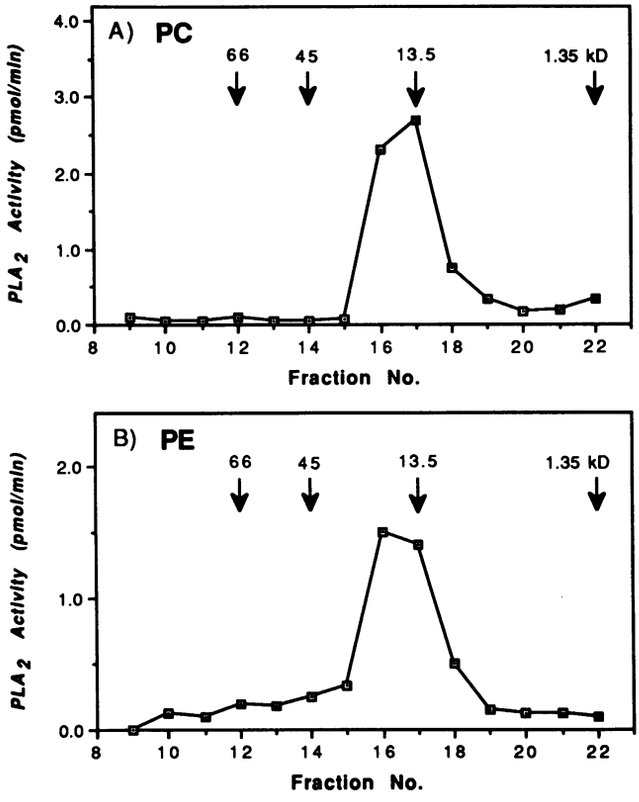

Figure 2. Fractionation of kidney mitochondrial extracts by FPLC Superose 12 gel filtration. Fractionation conditions were exactly the same as those used for the cytosolic extracts (Fig. 1). PLA_2 activity was measured using (*A*) PC or (*B*) PE as substrates. Molecular mass markers were the same as those described in the legend to Fig. 1.

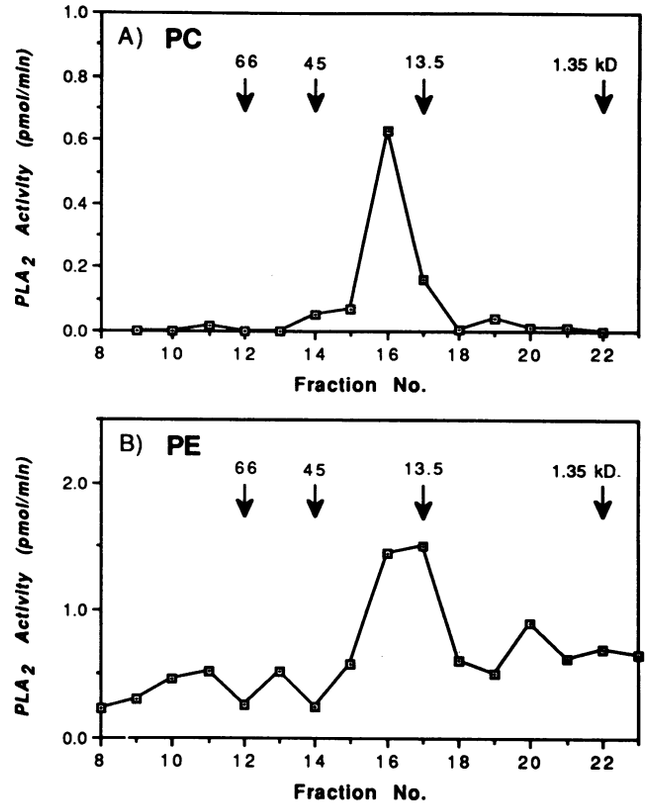

Figure 3. Fractionation of kidney microsomal extracts by FPLC Superose 12 gel filtration. Fractionation conditions were identical to those used for cytosolic (Fig. 1) and mitochondrial (Fig. 2) extracts. PLA_2 activity was measured using (*A*) PC or (*B*) PE as substrates. Molecular mass markers were the same as those described in the legend to Fig. 1.

Statistics. All values are presented as the mean±1 SE of the mean. Statistical significance was evaluated using the Student's *t* test or analysis of variance with $P < 0.05$ regarded as significant.

Results

Cytosolic, mitochondrial, and microsomal forms of PLA_2. To partially characterize kidney subcellular PLA_2 activity, cytosolic, mitochondrial, and microsomal samples were fractionated by Superose 12 gel filtration chromatography. Activity directed against PC in the freshly prepared cytosolic fraction migrated as a single peak with mobility characteristics corresponding to an apparent molecular mass of 55 kD (Fig. 1 *A*). This is similar to the previously described peak of cytosolic activity (17) which we have subsequently found to have an actual molecular mass of approximately 110 kD as determined by SDS-PAGE (19). When PE was used as a substrate there were two peaks of activity (Fig. 1 *B*), one migrating in the same position as that seen with PC and one migrating with much smaller standards (at ~ 14 kD).

Mitochondrial PLA_2 activity migrated as a single peak, eluting in fractions 16 and 17, with an estimated molecular mass of 14 kD. No difference was seen in the position of the peak of activity whether PC or PE was used as substrates (Fig. 2).

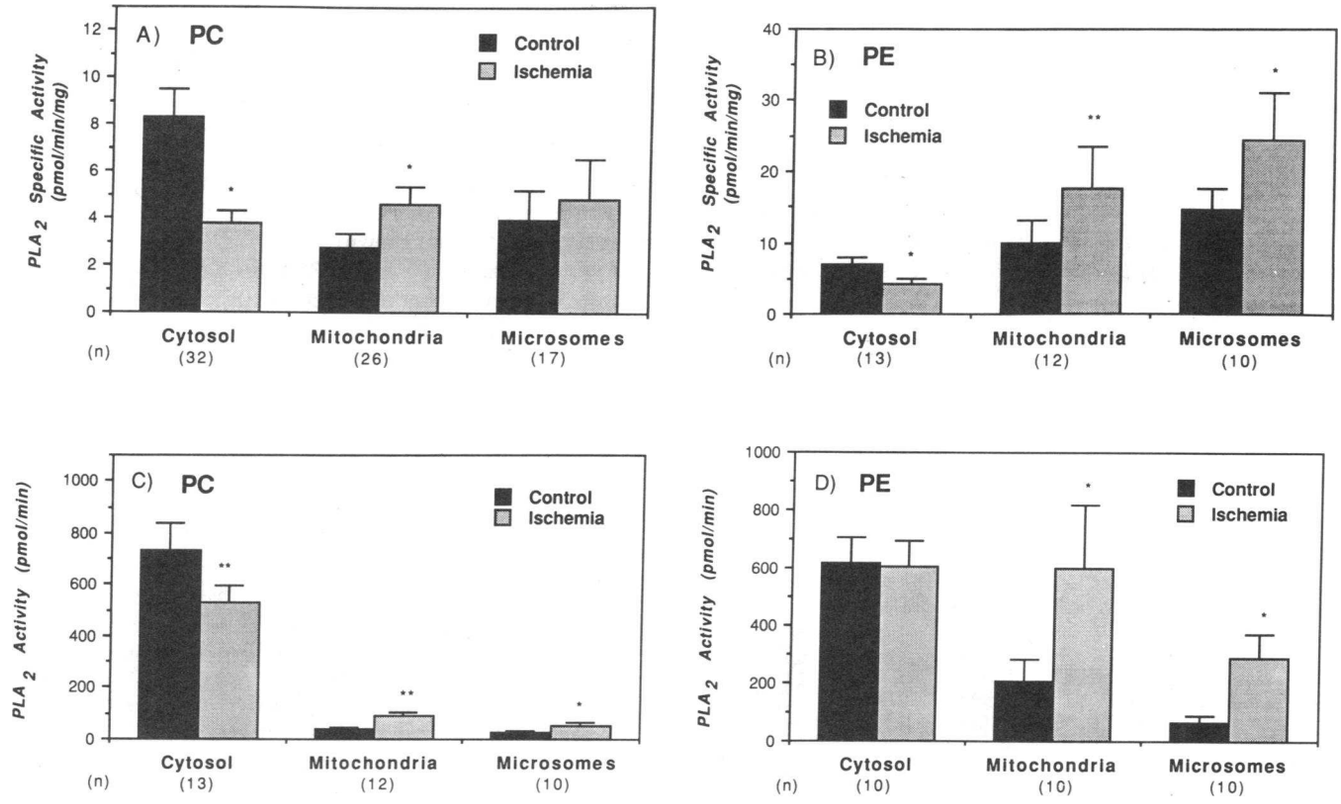

Figure 4. Cytosolic, mitochondrial, and microsomal PLA_2 (*A* and *B*) specific and (*C* and *D*) total activities in control (contralateral kidney) extracts and extracts of kidneys after 45 min of ischemia without reperfusion. There were significant reductions in cytosolic PLA_2 specific activity when either (*A*) PC or (*B*) PE were used as substrates. There was greater specific activity against PE than PC in the mitochondrial and microsomal extracts. Ischemia resulted in increased mitochondrial PLA_2 specific activity against PC and PE and increased microsomal activity against PE. Cytosolic total PLA_2 activity was significantly decreased when PC was used as substrate (*C*). By contrast, mitochondrial and microsomal total PLA_2 activities were enhanced with ischemia, whether PLA_2 activity was measured using (*C*) PC or (*D*) PE as substrate. $^*P < 0.05$; $^{**}P < 0.01$ compared with controls.

The microsomal extract contained a single peak of activity. This PLA_2 activity eluted from the column at exactly the same position as the mitochondrial PLA_2 activity against both PC and PE (Fig. 3).

When PLA_2 activities in control kidney subcellular fractions were compared, the highest PLA_2 specific (Fig. 4 *A*) and total activities (Fig. 4 *C*) against PC were observed in the cytosolic fraction. While the specific PLA_2 activity of the cytosol was approximately the same whether PC or PE were used as substrates, the mitochondrial and microsomal fractions had greater specific activities against PE (Fig. 4, *A* and *B*).

Subcellular distribution of PLA_2 activity in the kidney after ischemia without reflow. To determine whether PLA_2 activity in the various subcellular fractions was altered with ischemia, the activity of mitochondria, microsomes, and the 100,000-g supernatant (cytosol) were assayed after varying periods of ischemia without reperfusion. To establish that there was no differential extraction between control and ischemic kidneys, owing to ischemia-related membrane damage, cytosolic activity was determined as a fraction of total (cytosolic + mitochondrial + microsomal) activity in three initial experiments using PC as substrate. The fraction of total activity in the high speed supernatant was 78±4% in controls and 73±7% in ischemic kidneys.

Ischemia without reperfusion resulted in a rapid decrease of cytosolic PLA_2 specific activity when either PC or PE were used as substrates (Fig. 4, *A* and *B*). Decreases of both specific and total activities against PC were seen as soon as 5 min after placing the clamp (Fig. 5). Contralateral control kidney soluble PLA_2 activity was equal to that of kidneys in sham-operated animals (data not shown). Cytosolic PLA_2 activity was determined in the presence and absence of calcium (either no added calcium or 4 mM Ca^{2+} in the reaction buffer containing 0.75 mM EGTA and 0.75 mM EDTA. As depicted in Fig. 5 the enzymatic activity against PC was Ca^{2+} dependent. The decreased cytosolic enzymatic activity, with ischemia alone, was observed when the assay was performed in the presence or absence of Ca^{2+}.

In contrast to the decrease in cytosolic PLA_2 specific activity, 45 min of ischemia resulted in a significant increase of specific activity in the mitochondrial fraction when activity was determined against PC (Fig. 4 *A*) and in both mitochondrial and microsomal fractions when PE, the preferred substrate for PLA_2 in these fractions, was used (Fig. 4 *B*). Whereas total cytosolic PLA_2 activity was decreased when PC was used as substrate, total activity of both mitochondrial and microsomal fractions were increased in the ischemic kidney whether PE or PC was the substrate (Fig. 4, *C* and *D*). Ischemic mitochondria were also found to have higher PLA_2 activity when PC was used as substrate (1.5±0.2 as compared to 0.8±0.4 pmol/min per mg protein, $n = 5$), when 5 mM cholate was used

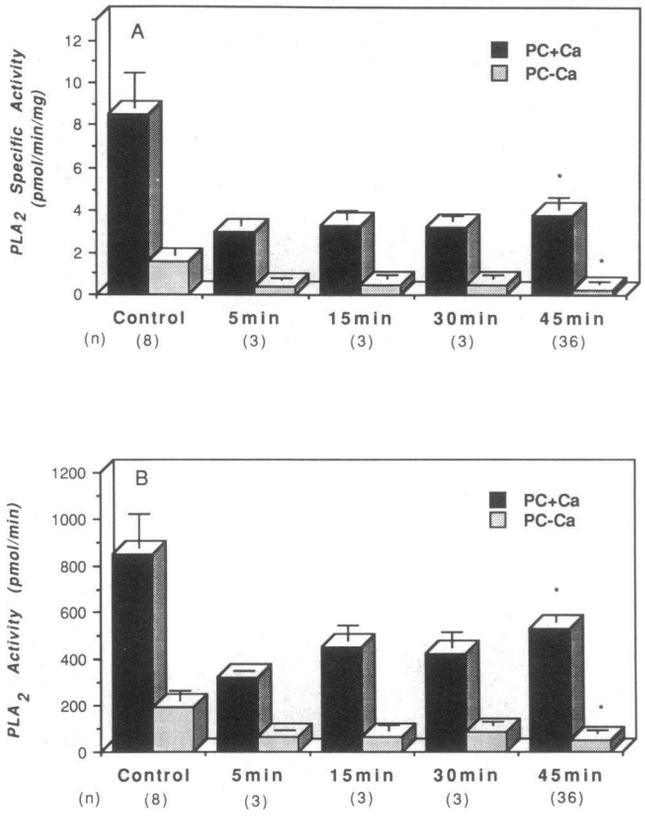

Figure 5. Cytosolic PLA_2 (*A*) specific and (*B*) total activity in extracts taken from kidneys after varying periods of ischemia without reperfusion. The assay was performed using a buffer containing 0.75 mM EGTA and 0.75 mM EDTA with (*PC + Ca*) or without (*PC − Ca*) 4 mM Ca^{2+}. *n* = number of experiments. $*P < 0.05$ compared with controls.

to extract activity. This difference was observed even though cholate extraction resulted in recovery of only 36.0±8.8% (*n* = 6) of KCl-extracted activity.

PLA_2 activity after 45 min of ischemia and 1 h of reflow. To determine whether PLA_2 activity was altered after reperfusion of the ischemic kidney, cytosolic and mitochondrial PLA_2 activities were measured after 45 min of ischemia followed by 1 h of reperfusion. As demonstrated in Fig. 6, there were significant increases of cytosolic PLA_2 specific and total activities against both PE and PC with ischemia and reperfusion. Mitochondrial specific activity against PC was increased. Mitochondrial and microsomal specific and total activities against PE were increased with ischemia and reperfusion.

Characterization of the cytosolic and mitochondrial PLA_2 activity after reflow. To further characterize PLA_2 activation associated with ischemia and reflow, cytosolic and mitochondrial samples were matched for protein and fractionated by Superose 12 gel filtration chromatography. PLA_2 activity measured against PC in ischemic/reperfusion cytosolic fractions migrated as a single peak (Fig. 7 *A*) at the same position as the peak of activity in control kidney cytosol (Fig. 1 *A*). When PE was used as substrate, three peaks of activity were seen, eluting at fractions 13, 17, and 21 (Fig. 7 *B*). The first two peaks correspond to the peaks of activity observed in control kidney cytosolic fractions when PE is used as substrate (Fig. 1 *B*). The third peak, eluting with an apparent molecular mass of ~ 2–3 kD, was not present in the preischemic samples.

Mitochondrial ischemia/reperfusion samples, when fractionated by Superose 12 gel filtration chromatography, had two peaks of activity against PC and PE (fractions 17 and 21) (Fig. 8), whereas only one peak (fraction 17) was present in control mitochondrial samples (Fig. 2 *B*). Microsomal fractionation resulted in a pattern of PLA_2 activity similar to that of the mitochondria (data not shown).

Cytosolic and mitochondrial PLA_2 pH dependency. In both cytosolic and mitochondrial fractions from control kidneys, the PLA_2 activities had pH optima in the range 8.5–9.5, whether PC (Fig. 9) or PE (data not shown) was used as substrate. Ischemia and reperfusion did not alter the pH optima of cytosolic (data not shown) or mitochondrial PLA_2 activity (Fig. 9 *B*).

Calcium dependency of cytosolic and mitochondrial forms of kidney PLA_2. PLA_2 activity in each of the cellular fractions is Ca^{2+} dependent. As shown in Fig. 5, cytosolic activity against PC and PE is markedly enhanced with calcium. The specific Ca^{2+} concentration dependency of cytosolic and mitochondrial PLA_2 activity is shown in Fig. 10. This calcium dependency of cytosolic and mitochondrial fractions was unaffected by ischemia alone or ischemia and reperfusion (data not shown). Note that cytosolic PLA_2 activity was enhanced when Ca^{2+} concentration was increased from 100 nM, a value representing baseline cytosolic Ca^{2+} concentration in renal epithelial cells (20), to 200–300 nM, values well within the range reached in response to both physiological and pathophysiological stimuli (1). By contrast, mitochondrial PLA_2 activity was not increased until $[Ca^{2+}]$ was increased to levels > 1 μM. Activity increased, however, as $[Ca^{2+}]$ increased to 5 μM, a value well within the range of variation of mitochondrial matrix $[Ca^{2+}]$ under physiological and pathophysiological conditions (21).

Discussion

Although a considerable amount is known about secretory forms of PLA_2 the intracellular forms are less well characterized. Our results indicate the presence of two distinct forms of PLA_2 in the cytosolic fraction of the kidney. The large molecular weight form has been previously characterized and recently purified in our laboratories (17, 19). This form is active against PE and PC. In addition to this form, our data indicate the presence of an additional smaller molecular weight form that has activity against PE but not PC. It is unlikely that this smaller form is a contaminant from the mitochondria or microsomes since the smaller molecular weight forms extracted from mitochondria and microsomes have activity against both PC and PE.

In both mitochondrial and microsomal fractions from control kidneys there were single peaks of activity with apparent molecular mass of ~ 14 kD. This size, together with the alkaline pH optimum and the Ca^{2+} dependency, are characteristics similar to those of previously described mitochondrial and microsomal forms from other tissues, such as rat liver (22, 23) and spleen (24). Membrane (mitochondrial and microsomal)-associated PLA_2 activity was solubilized with 1 M KCl, indicating that the enzymes existed as membrane-associated forms rather than as integral membrane proteins. This result is similar to that of Aarsman et al. (25), who found that total platelet lysate PLA activity was recovered in 1 M KCl extracts.

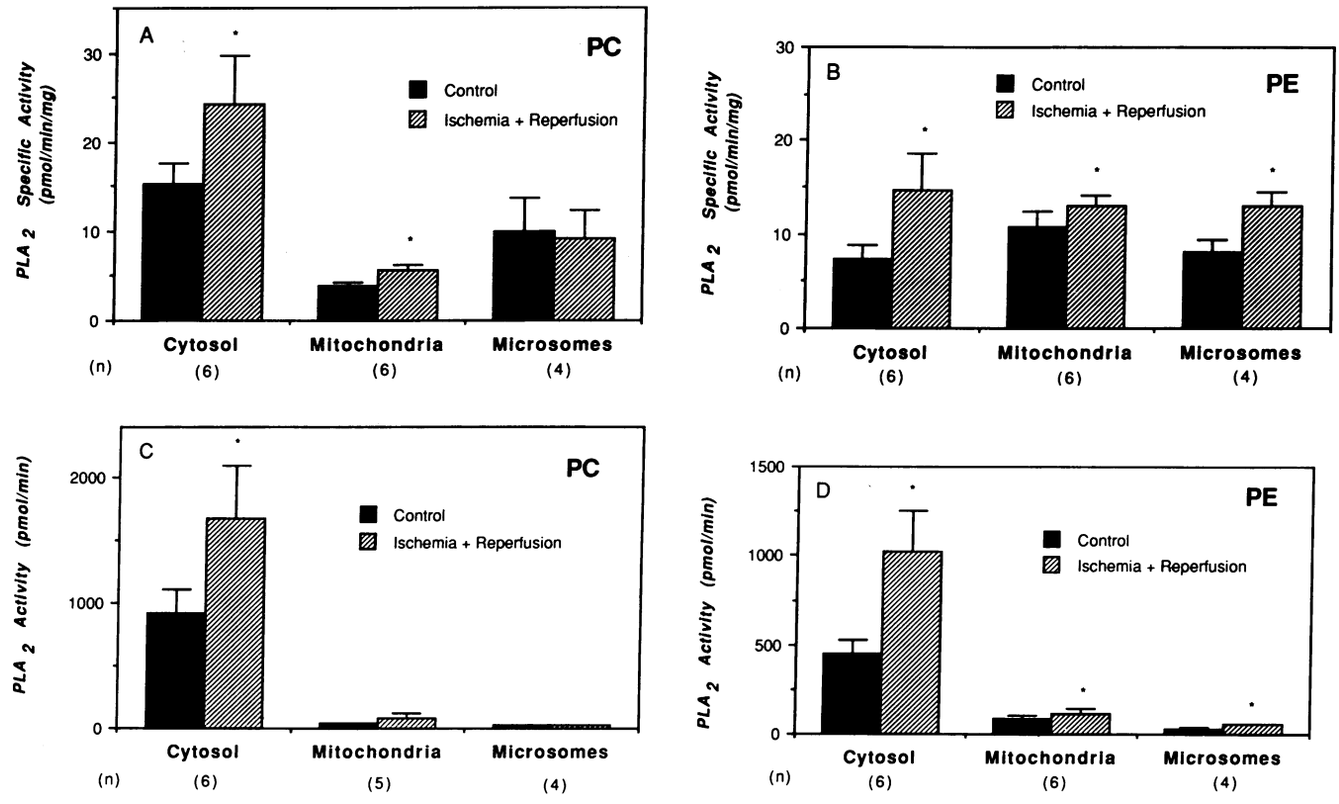

Figure 6. Cytosolic, mitochondrial, and microsomal PLA_2 (*A* and *B*) specific and (*C* and *D*) total activities in control (contralateral kidney) extracts and extracts of kidneys removed after 45 min of ischemia and 1 h of reperfusion. Cytosolic and mitochondrial PLA_2 specific activities were increased in the postischemic kidney extracts when PC was the substrate (*A*). Cytosolic, mitochondrial, and microsomal extract specific PLA_2 activities were increased when PE was the substrate (*B*). Total PLA_2 activity was enhanced with ischemia in the cytosolic fraction when PC was the substrate (*C*) and in cytosolic, mitochondrial, and microsomal fractions when PE was the substrate (*D*). $^*P < 0.05$ compared with controls.

The activities from cytosol, mitochondria, and microsomes were Ca^{2+} dependent with alkaline pH optima. When the detailed Ca^{2+} concentration dependency of cytosolic and mitochondrial PLA_2 activities was compared, an important difference was noted. The cytosolic form of the enzyme was activated at Ca^{2+} concentrations at least one order of magnitude lower than those necessary to enhance mitochondrial PLA_2 activity. The $[Ca^{2+}]$ sensitivity characteristics are consistent with important physiological and pathophysiological roles for both cytosolic and mitochondrial enzymes in the intact cell. The similarities between the $[Ca^{2+}]$ sensitivity of the cytosolic form and the $[Ca^{2+}]$ sensitivity of prostaglandin production in mesangial cells which we previously reported (26), together with the fact that the major form of PLA_2 activity in the mesangial cell and kidney cytosolic extracts is identical (17), indicates that this form is activated with changes in cytosolic $[Ca^{2+}]$ seen with physiological (18) as well as ischemic (27, 28) and anoxic influences (29). Furthermore, even though the mitochondrial form is at least one order of magnitude less sensitive to calcium, basal matrix Ca^{2+} concentrations have been estimated at 0.5–1.5 μM (21), and kidney mitochondria accumulate a great deal of Ca^{2+} during hypoxia (30). Since matrix free $[Ca^{2+}]$ is highly correlated with extramitochondrial free $[Ca^{2+}]$ (31) and total mitochondrial Ca^{2+} (21), it is very likely that matrix free $[Ca^{2+}]$ increases with ischemia and reperfusion to levels at which mitochondrial PLA_2 enzymatic activity is enhanced.

Phospholipid degradation is believed to contribute to tissue injury associated with ischemia in the kidney (32), brain (11), heart (4, 33), intestine (34), and liver (35). This observation, together with experiments demonstrating arachidonic acid release or prostaglandin synthesis, is consistent with the conclusion that phospholipase A_2 may be an important contributor to ischemic cell damage. Other enzymatic processes, however, besides PLA_2 activity directed against phospholipids, can result in elevated tissue free fatty acid levels (13). For example, phospholipase C, followed by diacylglycerol lipase, or followed by diacylglycerol kinase and phosphatidic acid-specific PLA_2, can result in increased free fatty acid levels, as can increased triglyceride lipase activity. PLA_1 followed by lysophospholipase, can also generate increased free arachidonate levels. Plasmalogenase and lysophospholipase can release arachidonate from plasmalogens. Furthermore, inhibition of reacylation mechanisms can also enhance tissue levels of free fatty acids. For example, the activity of lysophosphatidylcholine acyltransferase has been reported to be decreased in ischemic pig myocardium (36).

PLA_2 enzymatic activity has seldom been directly measured during ischemia or after reperfusion. It has been generally assumed that increases in cytosolic Ca^{2+} concentration brought about by ischemia will activate a calcium-dependent PLA_2 enzyme. There are many forms of PLA_2, however, and some of these forms in nonrenal tissues are not Ca^{2+} dependent (37). Furthermore, there are additional ways to activate PLA_2 other than by increasing $[Ca^{2+}]$ (13).

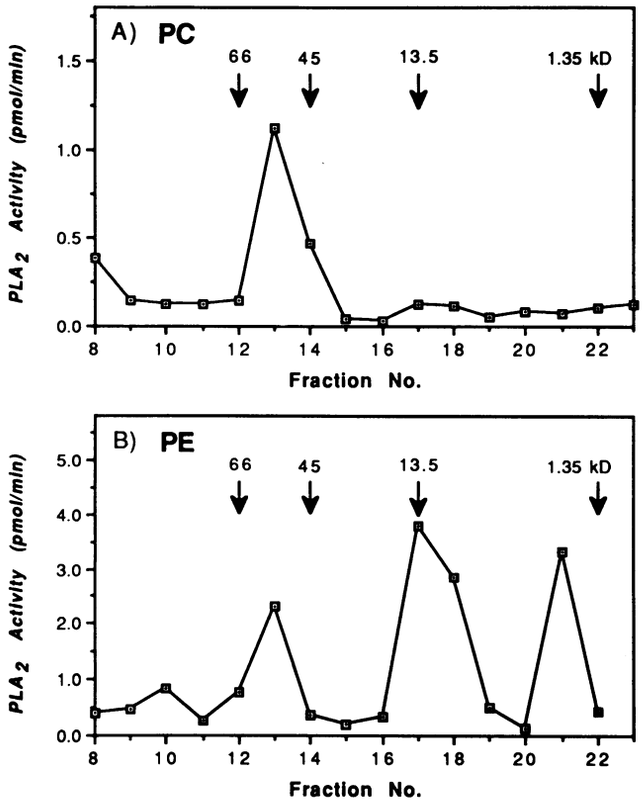

Figure 7. Fractionation of cytosolic extracts of kidneys exposed to 45 min of ischemia followed by 1 h of reperfusion. Fractionation was performed by FPLC Superose 12 gel filtration. Fractions were assayed for PLA_2 activity using either (*A*) PC or (*B*) PE as substrates. Molecular mass markers are those described in the legend to Fig. 1.

Direct experimental evidence for the activation of phospholipases with ischemia was found in extracts of gerbil brain tissue taken after 1 min of bilateral carotid occlusion (38); however, PLA_2 activity returned to baseline levels by 5 min of ischemia and there was no correlation between PLA_2 activity and the tissue levels of free fatty acid during and after ischemia. PLA_2 activity was increased in homogenates of small intestinal mucosal cells after ischemia and reperfusion (34). Kawaguchi and Yasuda (39) reported increases of mitochondrial and microsomal PLA_2 activity in hypoxic myocardium. This PLA_2 activity had a substrate specificity for PE, but the mechanism of activation was not clarified and the enzymatic activity was not further characterized. By contrast, in the ischemic area of the isolated rat heart homogenate and mitochondrial PLA_2 enzymatic activity was found to be decreased 20 min after coronary artery ligation (14).

In the present study PLA_2 activity against PE and PC in the cytosol decreased rapidly after ischemia if the kidney was not reperfused. Our data do not provide an explanation of decreased cytosolic activity after ischemia without reperfusion. Endogenous inhibitors of PLA_2 have been found in other systems (40) and it is possible that inhibitors, such as unsaturated fatty acids (41), may be present in the cytosolic fraction after 45 min of ischemia. Bentham and colleagues (14) attributed the decrease in PLA_2 activity seen after 20 min of ischemia in the heart to the presence of lysophosphatidylcholine and palmitoylcarnitine, two amphiphiles that accumulate in the ischemic myocardium and inhibit PLA_2 in vitro. The decreased cytosolic PLA_2 activity may limit tissue damage during the ischemic period. In contrast to cytosolic activity, mitochondrial and microsomal activities were increased at the end of the ischemic period. Bentham et al. (14) found a decrease in mitochondrial PLA_2 activity after 20 min of ischemia but no differences in sarcolemmal PLA_2 activities comparing ischemic and control hearts.

Increased mitochondrial PLA_2 activity may have important consequences for tissue injury secondary to ischemia. Mitochondrial membranes are composed of phospholipids with 65% of fatty acids having unsaturated bonds, thus making them particularly good substrates for PLA_2 action (42). Minimal phospholipid degradation is sufficient to produce a modification of inner membrane permeability properties (7, 43). Inability to reverse mitochondrial dysfunction upon reperfusion correlates with inability to reverse ischemic cell injury (44). The alkaline pH optima of PLA_2 activity in the mitochondrial and cytosolic compartments may explain the protection afforded by acidosis of kidneys tubules exposed to anoxia (45).

When ischemia was followed by reperfusion, cytosolic, mitochondrial, and microsomal PLA_2 activities were increased. The PLA_2 activity of cytosol had essentially the same molecular weight, pH optimum, and Ca^{2+} dependency under control or postischemic conditions. We found a different, smaller form of mitochondrial PLA_2 activity when PE was used as a substrate (M_r = 2–3 kD). This "smaller form" may represent a protein modification of a larger form which results in increased interaction between the protein and the gel filtration column.

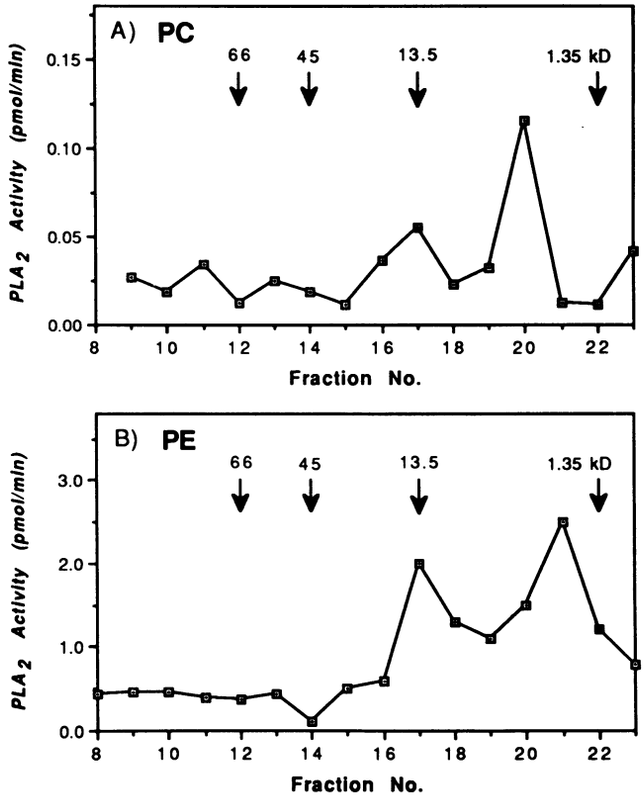

Figure 8. Fractionation of mitochondrial extracts of kidneys exposed to 45 min of ischemia followed by 1 h of reperfusion. Fractionation was performed by FPLC Superose 12 gel filtration. Fractions were assayed for PLA_2 activity using either (*A*) PC or (*B*) PE as substrates. Molecular mass markers are those described in the legend to Fig. 1.

We have previously shown, for example, that the form of PLA_2 that migrates with an M_r of ~ 55 kD has a molecular mass of 110 kD on SDS-PAGE when purified (19). This may be related to interaction of hydrophobic domains on PLA_2 with the Superose 12 column (19). A less likely, but not excluded, possibility is that the 2–3-kD form represents a breakdown product of the prevalent mitochondrial form, perhaps peptide fragments with residual PLA_2 activity.

While elevated $[Ca^{2+}]$ may be important for activation of the PLA_2 enzymes in vivo with ischemia the preservation of enhanced activity in vitro, under conditions where $[Ca^{2+}]$ is fixed, suggests a stable modification of the enzymes. Our observations suggest that ischemia and reperfusion may result in covalent modifications of the enzymes and indicate that there may be continued PLA_2 activation even if $[Ca^{2+}]$ levels return to baseline values. This may explain, for example, why arachidonic acid continues to accumulate in heart tissue after reperfusion (15). PLA_2 may be regulated via phosphorylation by protein kinase C as previously suggested (17, 26). There is evidence that protein kinase C is activated with ischemia (46). There is also evidence for enhanced Ca^{2+}-phosphatidylserine-independent kinase activity with ischemia (47). It is, therefore, possible that phosphorylation of the enzyme can occur after ischemia and reperfusion, resulting in enhanced activity. It is also possible that the decreased cytosolic PLA_2 activity that we found after ischemia without reoxygenation may be due to dephosphorylation of PLA_2. Alternatively, PLA_2 could be tightly associated with regulatory proteins. Protein kinase C has been suggested to phosphorylate proteins which inhibit

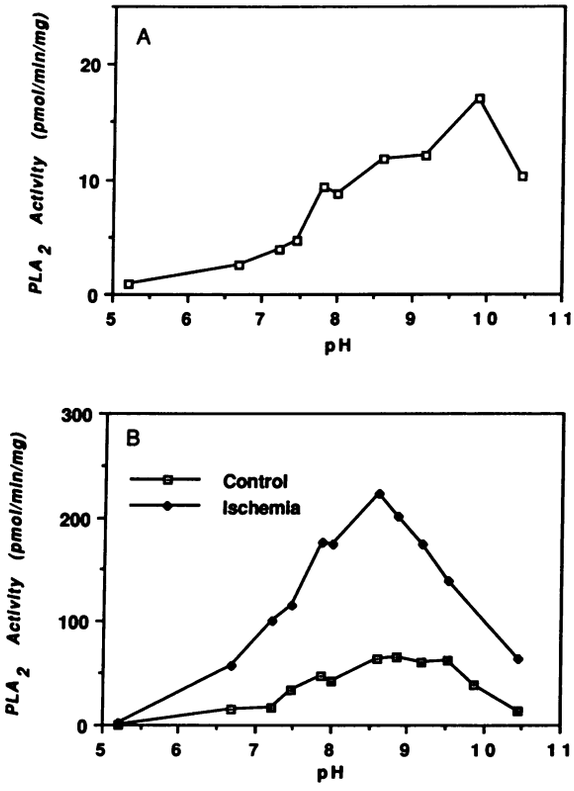

Figure 9. pH dependency of (*A*) cytosolic and (*B*) mitochondrial PLA_2 activity. PLA_2 activity was determined using PC as the substrate. Both cytosolic and mitochondrial fractions have alkaline pH optima. Ischemia does not alter the pH optimum of the mitochondrial activity (*B*), nor does it alter the cytosolic PLA_2 pH optimum (data not shown).

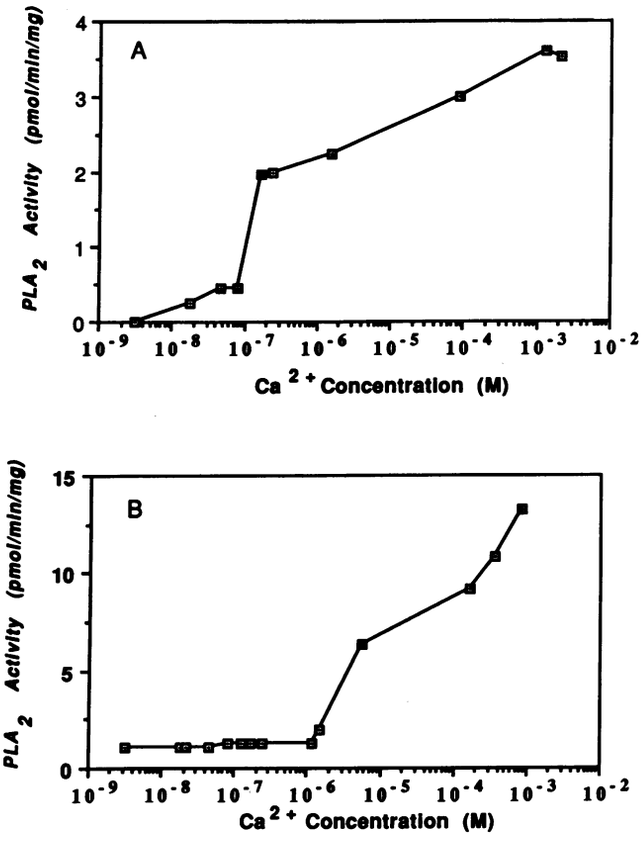

Figure 10. Ca^{2+} dependency of (*A*) cytosolic and (*B*) mitochondrial PLA_2 activity. Activity was determined using PC as the substrate. Cytosolic PLA_2 activity (*A*) increased when $[Ca^{2+}]$ was increased over the physiological range (0.1–1.0 μM). Mitochondrial PLA_2 activity was at least one order of magnitude less sensitive to $[Ca^{2+}]$. Nevertheless mitochondrial PLA_2 activity increased over a range (1.0–10.0 μM) that mitochondrial matrix $[Ca^{2+}]$ is likely to vary under physiological and pathophysiological conditions.

PLA_2 by binding to the phospholipid substrate (48). Ischemia and reperfusion may result in modifications of these proteins and hence alter PLA_2 activity in this manner.

In conclusion, we have demonstrated that different forms of PLA_2 are prevalent in the cytosolic and membrane (mitochondrial and microsomal) compartments of the rat kidney. These forms differ markedly in apparent molecular weight as indicated by migration patterns by gel filtration chromatography. The $[Ca^{2+}]$ sensitivity of both cytosolic and mitochondrial PLA_2 activities indicate that the enzymes are likely regulated by Ca^{2+} in vivo. Ischemia without reperfusion results in increased PLA_2 activity in mitochondrial and microsomal fractions. After ischemia and reperfusion, cytosolic, mitochondrial, and microsomal PLA_2 activities were enhanced and a new form of enzymatic activity was present in the mitochondrial fraction. These stable modifications of enzymatic activity cannot be explained by changes in $[Ca^{2+}]$ and suggest other regulatory influences. Enhanced PLA_2 activity may play an important role in the mediation of cellular injury after an ischemic insult.

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