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Cytosolic Phospholipase A_2 (PLA_2), but Not Secretory PLA_2, Potentiates Hydrogen Peroxide Cytotoxicity in Kidney Epithelial Cells*

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Phospholipase A_2 (PLA_2) and reactive oxygen species have been implicated both individually and synergistically in various forms of cellular injury. The form(s) of PLA_2 important for cell injury and the implications of enhanced activity of the enzyme, however, have not been discerned. Previous studies reveal an increase in PLA_2 activity associated with cell injury, but this association does not establish a causal relationship between the increase in activity and the injury. LLC-PK_1 cell lines were created that express either the cytosolic PLA_2 or a group II PLA_2. The susceptibility of these cells to hydrogen peroxide toxicity was determined in order to evaluate the relative importance of these two forms of PLA_2 in oxidant injury. Expression of cytosolic PLA_2 in the LLC-cPLA$_2$ cell line was associated with a 50-fold increase in PLA_2 activity in the cytosolic fraction, an increase in agonist-stimulated arachidonate release, and immunodetection of the cytosolic PLA_2 protein that was undetectable in control cells. Exposure to hydrogen peroxide or menadione, but not mercuric chloride, resulted in significantly greater lactate dehydrogenase release in LLC-cPLA$_2$ cells when compared with control cells. Exogenous arachidonic acid (150 μM) did not enhance hydrogen peroxide-induced injury. The intracellular calcium chelator, 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid/tetra(acetoxymethyl) ester, protected the cells against injury, but the calcium ionophore, A23187, did not increase injury. Glycine conferred no protective effect against hydrogen peroxide toxicity. By contrast to these results with cytosolic PLA$_2$-expressing cells, secretory PLA_2 expression to very high levels did not increase susceptibility to hydrogen peroxide. Thus, cytosolic PLA_2 may an be an important mediator of oxidant damage to renal epithelial cells.

Phospholipase A_2 (PLA_2)[1] and reactive oxygen species have been implicated in various forms of cellular injury (1–3). PLA_2 activation may adversely affect cell viability by direct actions on membranes or indirectly due to metabolic products produced by the activity of this family of enzymes. PLA_2 can cause membrane degradation and changes in plasma and mitochondrial membrane bioenergetics and permeability (4–6). These effects, together with increased production of lysophospholipids, arachidonic acid, eicosanoids, platelet-activating factor, and reactive oxygen species (7) due to increased PLA_2 action, have been implicated in destructive cellular processes in kidney (4, 8–10), heart (11), intestine (12), and central nervous system (13–15).

We have proposed that PLA_2 can act synergistically with reactive oxygen species to cause cellular injury due to enhanced susceptibility of peroxidized membranes to the action of PLA_2 (4). Reactive oxygen species are considered to be important mediators of cytotoxicity in a variety of situations, including ischemia/reperfusion (16), toxic cellular injury (17), and apoptosis (18, 19). Oxidant stress and peroxidation of lipid substrates have been shown to enhance PLA_2 activity (2, 14, 15), and the associated activation of PLA_2 has been proposed to be a critical factor in injury (2, 15). Despite such evidence, the role of PLA_2 in cellular injury remains controversial (20). PLA_2 has been shown to be protective in some settings of lipid peroxidation and hypoxia (21–23). Furthermore, since many different forms of PLA_2 exist (24, 25), a clarification of the importance of PLA_2 in cell injury requires the recognition of the role played by each of the various forms of the enzyme (25) present in the cell.

The 85-kDa cytosolic PLA_2 (cPLA$_2$) may be of particular importance as a mediator of cellular injury. This enzyme has a high specificity for arachidonic acid at the *sn*-2 position of phospholipids and is translocated to its site of action, lipid membranes, in response to increases in cytosolic calcium concentrations in the nanomolar range (26). Cytosolic calcium concentrations increase in many pathobiological states, such as those associated with cellular ATP depletion, toxin, and reactive oxygen species exposure. Hence, activation of this enzyme would be expected under various conditions in which cells are injured. PLA_2 activation with arachidonic acid release is associated with membrane degradation and changes in plasma and mitochondrial membrane bioenergetics and permeability (4–6). Enzymes that metabolize arachidonic acid produce reactive oxygen species with destructive potential (27). Oxygenated metabolites of arachidonic acid may themselves be toxic (28), as may the lysophospholipids that result from cPLA$_2$ action on phospholipids. The amphiphilic lipid molecules that are generated by PLA_2 action can have direct detergent actions on the

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[1] The abbreviations used are: PLA_2, phospholipase A_2; cPLA$_2$, cytosolic PLA$_2$; sPLA$_2$, group II PLA$_2$; BAPTA-AM, 1,2-*bis*-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid/tetra(acetoxymethyl) ester; DMEM, Dulbecco's modified Eagle's medium; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; PC, 1-stearoyl-2-arachidonyl phosphatidylcholine; PMA, 12-myristate 13-acetate; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; MDCK, Madin-Darby canine kidney cells.

membrane and can influence the activity of membrane channels and/or proteins. We have proposed that cPLA$_2$ plays an important role in cell injury in the post-ischemic kidney and brain (8, 14).

In contrast to cPLA$_2$, the 14-kDa group II PLA$_2$ (sPLA$_2$), found in many organs and cell types, is a prototypical interfacial enzyme that is secreted from cells, has no selectivity for the fatty acid in the *sn*-2 position, and requires higher calcium concentrations for activation (29). It has characteristics in common with snake venom group II PLA$_2$ and is found in inflammatory exudates (30); however, its role in cytotoxicity is undefined.

Other forms of PLA$_2$ are less well characterized. Some have proposed that calcium-independent forms are important for cell injury (9). The structural characteristics of these calcium-independent forms have not been reported to date.

To better define potential roles of cPLA$_2$ and sPLA$_2$ in direct cytotoxicity and to explore the complex interrelationship between PLA$_2$ and reactive oxygen species in cellular injury, we examined the effect of PLA$_2$ expression on hydrogen peroxide-induced injury in renal epithelial cells that normally express low amounts of PLA$_2$ activity. LLC-PK$_1$ cell lines were created that stably express cPLA$_2$ (31) or the 14-kDa group II PLA$_2$, sPLA$_2$ (30). When exposed to H_2O_2 or menadione, the cPLA$_2$, but not sPLA$_2$, overexpressing cells were much more susceptible to cell death than control cells. Of the potentially injurious agents examined, this predisposition to injury appeared to be specific for H_2O_2-mediated toxicity, because cPLA$_2$ expressing cells did not demonstrate enhanced injury secondary to mercuric chloride or A23187. The increased susceptibility to H_2O_2 injury was mitigated by pretreatment of the cells with BAPTA-AM, a chelator of intracellular calcium, but not with glycine, an agent found to protect kidney cells against various forms of injury (32).

EXPERIMENTAL PROCEDURES

Materials—A23187, $HgCl_2$, phorbol 12-myristate 13-acetate (PMA), β-NAD, β-NADPH, glutathione, glutathione reductase, xanthine, xanthine oxidase, 5,5′-dithiobis(2-nitrobenzoic acid), lithium lactate, menadione, and [^{3}H]arachidonic acid were purchased from Sigma. H_2O_2 and glycine were obtained from Fisher. The aminoglycoside antibiotic, G418 sulfate, was from Life Technologies, Inc. Bovine serum albumin fraction V (BSA) was from Boehringer Mannheim. 1-Stearoyl-2-[1-^{14}C]arachidonyl phosphatidylcholine ([^{14}C]PC) was from Amersham Corp. The acetoxymethyl form of the intracellular Ca^{2+} chelator 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid/tetra(acetoxymethyl) ester (BAPTA/AM) and hygromycin B were obtained from Calbiochem. The cell permeant form of the fluorescent H_2O_2 indicator 5-(and -6)-carboxy-2′,7′-dichlorodihydro-fluorescein diacetate was obtained from Molecular Probes (Eugene, OR). The WAKO NEFA-C fatty acid detection kit was purchased from Biochemical Diagnostics Inc. (Edgewood, NY). 1-Palmitoyl,2-oleoyl-phosphatidylglycerol was obtained from Avanti Polar Lipids (Alabaster, AL). Protein measurements based on Bradford's assay were performed with reagents from Bio-Rad.

Cell Culture—LLC-PK$_1$ cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM containing 4.5 g/liter glucose and supplemented with 1% L-glutamine and 10% fetal calf serum and kept at 37 °C in 95% air, 5% CO_2 without antibiotics. Stable cell lines transfected with pcDNA I/Neo, pcDNA3, or pREP4 (Invitrogen, San Diego, CA) were maintained with 400 μg/ml G418 sulfate (pcDNA I/Neo or pcDNA3 transfected cells) or 500 μg/ml hygromycin B (pREP4 transfected cells). In preparation for experiments, cells were replated and grown in the absence of antibiotics to minimize any effect of these agents on the results. Small differences in culture conditions, cell passage number, and plating density may have resulted in some intrinsic variability between experiments. All experiments were, therefore, conducted with contemporaneous matched controls.

Plasmid Expression Systems—The mammalian expression construct containing human cPLA$_2$, pmt-PLA$_2$ (31), was a gift of Dr. Clark at Genetics Institute (Cambridge, MA). The plasmid pSQ140, containing human sPLA$_2$ (30), was the gift of Dr. Browning at Biogen Inc. (Cambridge, MA). The cDNA for human sPLA$_2$ was released from pSQ140 as the 835-base pair *Eco*RI/*Not*I restriction fragment. This fragment was cloned into the compatible restriction sites of the pcDNA3 mammalian expression vector to create pcDNA3/sPLA$_2$. The construct was sequenced using the Sequenase DNA Sequencing Kit from U. S. Biochemical Corp. The sequence and orientation of the insert in pcDNA3/sPLA$_2$ was compared with the published sequence (30).

Creation of Stable Transfectants—LLC-PK$_1$ cells were plated on 10-cm tissue culture plates at a density of approximately 250,000 cells/plate 2 days before transfection. The cells were transfected using the calcium phosphate technique as published (33). LLC-cPLA$_2$ cells were created by co-transfection of LLC-PK$_1$ cells with 2 μg of the G418 resistance plasmid pcDNA I/Neo and 18 μg of pmt-PLA$_2$. LLC-sPLA$_2$ cells were created by the transfection of LLC-PK$_1$ cells with 20 μg of pcDNA3/sPLA$_2$. LLC-vector cells were created by transfection with a neomycin or hygromycin resistance plasmid or with the pcDNA3 plasmid. There were no differences in characteristics of the vector control cells whether they carried the hygromycin or neomycin resistance plasmid. Two days after transfection the cells were trypsinized and transferred to medium containing G418 sulfate (400 μg/ml) for selection. Limiting dilutions of the transfected cells were made, and subclones with the highest cPLA$_2$ (LLC-cPLA$_2$) or sPLA$_2$ (LLC-sPLA$_2$) activity were selected and used for all further experiments.

Arachidonic Acid Release—Subconfluent cells in 12- or 24-well plates were labeled for 18–24 h with [^{3}H]arachidonic acid (0.2–0.3 μCi/ml) in serum-free Dulbecco's modified Eagle's medium (DMEM). After labeling, the medium was removed, and cells were washed with DMEM containing 0.2% BSA. To measure stimulated arachidonic acid release, cells were exposed to agonists or vehicle in DMEM, 0.2% BSA for 20–30 min at 37 °C in 95% air, 5% CO_2. The medium was removed and centrifuged, and the radioactivity in 250 μl of supernatant was measured in a liquid scintillation counter. In some experiments, cells were solubilized with 0.5 N NaOH or 1% Triton X-100, and the amount of [^{3}H]arachidonic acid released into the medium was expressed as a percent of total (cell-associated plus released).

PLA$_2$ Activity—Cells were washed with phosphate-buffered saline (PBS) and lysed by sonication (Heat Systems-Ultrasonics, Inc.) in a buffer containing 120 mM NaCl, 1 mM EDTA, and 50 mM Tris/HCl at pH 9.0. The lysate was centrifuged at 100,000 × *g* for 1 h at 4 °C. The supernatant was removed and stored at 4 °C. cPLA$_2$ activity was assayed at 37 °C for 20 min in 100-μl reactions that included 10 μl of [^{14}C]PC (final concentration 5 μM), 5 mM $CaCl_2$, 1 mM EDTA, 100 mM NaCl, and 75 mM Tris/HCl at pH 9.0. Reactions were quenched by adding 800 μl of Dole's reagent (32% isopropyl alcohol, 67% *n*-heptane, and 1% of 1 N H_2SO_4) (34) and vortexing. The sample was centrifuged for 2 min, and 400 μl of the upper phase was transferred to a new tube containing 600 μl of *n*-heptane and 50 mg of silica gel. After vortexing and allowing the silica gel to settle, 800 μl of supernatant was transferred to another tube containing 200 μl of *n*-heptane and 50 mg of silica gel. After vortexing and centrifuging, 800 μl of supernatant was counted for radioactivity in a liquid scintillation counter. In order to elute membrane-associated PLA$_2$ activity, the 100,000 × *g* pellet was resuspended, sonicated, and incubated at 4 °C for 1 h in a buffer of 50 mM Tris/HCl, pH 8.0, 1 mM EGTA, 1 mM EDTA, and 1 M KCl before repeating centrifugation (8). The cPLA$_2$ activity in 10 μl of the supernatant was assayed in a volume of 100 μl of the previously described cPLA$_2$ assay buffer. The protein concentrations in the cell fractions were determined using bovine serum albumin as a standard.

To measure sPLA$_2$ activity released from cells, tissue culture medium of 90–95% confluent cell monolayers was changed to serum-free DMEM for 24 h; after that the medium was collected and immediately centrifuged to remove any cellular debris. sPLA$_2$ activity was assayed using a detergent/phospholipid substrate as described in detail by Santos (35). Briefly, 10 μl of each sample was placed in a 96-well plate (Falcon) that had been pretreated with a solution of 1% gelatin in PBS and warmed to 37 °C. Fifty microliters of a substrate solution consisting of 0.35% w/v 2-oleoyl phosphatidyl glycerol in 0.4% w/v Nonidet P-40, 0.2% w/v sodium deoxycholate, 108 mM Tris/HCl pH 8.0, 10.8 mM $CaCl_2$, and 0.09 mM EDTA was added to each sample. The samples and substrate were incubated at 37 °C for 30 min. The reaction was quenched by the addition of 80 μl of color reagent A from the NEFA-C kit and incubated another 10 min, followed by the addition of 150 μl of color reagent B from the NEFA-C kit. After 10 min, the sample absorption was measured at 550 nm using an enzyme-linked immunosorbent assay plate reader. Specific activity was determined from standard curves created using dilutions of 1 mM oleic acid. Linearity of the test system over the range of the assay was documented using porcine pancreatic PLA$_2$ at known concentrations as a standard.

Immunoblotting—Immunodetection of cPLA$_2$ was performed as de-

scribed elsewhere (36). Briefly, crude extracts of proteins were separated by SDS-10% PAGE and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford MA). Nonspecific binding was blocked by preincubation with 5% nonfat dry milk in PBS containing 0.5% Tween 20 and 0.1% Thimerosal (Sigma). The membrane was exposed to 1:1000 diluted polyclonal antibody raised against porcine spleen cPLA$_2$ (36) and washed, and antibody binding was detected by using peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) and the chromogenic substrate, 4-chloro-1-naphthol.

Cytotoxicity Assays—In adherent cells grown in 12- or 24-well plates, cell death was determined by the fraction of the total lactate dehydrogenase (LDH) that had been released into the medium. Cells were tested when at least 80% confluent but before dome formation. All cells were fed with DMEM with 10% fetal calf serum the day before experiments. To test for H_2O_2 or A23187 toxicity, the medium was removed and replaced with serum-free DMEM, except for BAPTA/AM pretreatment experiments in which cells were incubated in nominally Ca^{2+}-free PBS. Cells were exposed to H_2O_2 or A23187 for 180 min or menadione for 90 min at 37 °C in 95% air, 5% CO_2 unless otherwise noted. The medium was then removed and centrifuged to remove any cells. The adherent cells were lysed with 1% Triton X-100 for 10 min, and this was added to any pellet from the centrifuged medium and vortexed. LDH activity in the medium and cell lysate was determined by adding 400 μl of the sample to 2.6 ml of 7 mM β-NAD in glycine/lactate buffer (58 mM lithium lactate and 200 mM glycine at pH 8.9) at 37 °C and measuring absorbance at 340 nm over 2 min.

Irreversible cell injury was also measured using trypan blue exclusion when so indicated. Cells were trypsinized, suspended in DMEM 10% fetal calf serum, centrifuged gently, and resuspended in serum-free DMEM. Cells were resuspended at 0.5–1.0 $\times$ 10^6/ml in 12-well plates and exposed to an agent of interest or vehicle at 37 °C in 95% room air and 5% CO_2. After the exposure, any adherent cells were gently scraped off, and the cell suspension was mixed with an equal volume of 0.4% trypan blue for 2 min before counting. Two hundred cells in random fields were counted, and cell death was reported as the percentage of all cells that took up trypan blue.

Antioxidant Enzyme Activities and Glutathione Content—Activities of three antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase) and total glutathione content were assayed using confluent cell monolayers in 10-cm cell culture plates. For each enzyme assay, the cells were washed twice in the appropriate buffer, scraped from the plate, centrifuged, resuspended in buffer, and lysed by sonication on ice. Cell lysates for catalase measurement were centrifuged for 30 min at 14,000 $\times$ *g* at 4 °C, and the supernatant was treated with 1/20th a volume of 1% Triton X-100 in buffer prior to measurement. Activities were normalized to cell protein.

Superoxide dismutase activity was assayed by spectrophotometric determination of the reduction of cytochrome *c* as described (37). In brief, the xanthine/xanthine oxidase reaction was performed in 50 mM potassium phosphate, 0.1 mM EDTA at pH 7.8 and 20 °C. Sample protein was added to achieve a 50% inhibition in the rate of superoxide-induced cytochrome *c* reduction. Assays were repeated with the addition of 1 mM potassium cyanide to distinguish Mn superoxide dismutase (resistant) from CuZn superoxide dismutase. Results are expressed in superoxide dismutase units/mg protein.

Catalase activity was measured, as described previously (38), by measuring the decomposition of H_2O_2. Absorbance at 240 nm was measured for 1 min after the addition of sample in a buffer containing 50 mM, pH 7.0, phosphate buffer and 10 mM H_2O_2. Results are expressed as mmol of O_2 produced per min for each ml of reaction normalized per mg of protein.

The glutathione peroxidase activity was determined using *t*-butyl hydroperoxide as a substrate and measuring the oxidation of NADPH at 340 nm (39). The assay was performed in a buffer containing 1 mM EDTA, 100 mM potassium phosphate, pH 7.7, with 0.5 mM *t*-butyl hydroperoxide, 150 μM NADPH, 5 mM glutathione, and 0.25 units of glutathione reductase. Sodium azide (1 mM) was added to the reaction to inhibit catalase activity. Nonenzymatic oxidation of NADPH and glutathione was measured by substituting buffer for sample, and this correction was made to all sample measurements. Glutathione-independent oxidation of NADPH, determined by conducting the assay without the addition of glutathione, was below the limits of detection. Results are expressed as milliunits/mg protein.

Total glutathione was measured in a protein-free cell lysate prepared by resuspending the cell pellet in 600 μl of 0.8% picric acid, sonicating on ice, and separating the protein precipitate by centrifugation at 14,000 $\times$ *g* for 5 min at 4 °C. Total glutathione was determined spectrophotometrically as described (40) using 5 μl of the supernatant in 1 ml of a buffer containing 0.2 mM NADPH, 100 mM sodium phosphate, pH 7.5, with 0.6 mM 5,5′-dithiobis(2-nitrobenzoic acid). After the addition of 0.1 unit of glutathione reductase, the increase in absorbance at 412 nm of each sample was recorded. Freshly prepared solutions of reduced glutathione were used to calibrate the assay, and results are reported as nanomoles of glutathione/mg of cellular protein.

Statistical Analysis—Values are presented as means ± 1 S.E. Significance was tested using Student's *t* test for paired data or analysis of variance where appropriate. Two-tailed *p* values < 0.05 were considered significant.

RESULTS

Stable Expression of cPLA$_2$ or sPLA$_2$ in LLC-PK$_1$ Cells—To confirm that LLC-PK$_1$ cells stably transfected with pmt-PLA$_2$ (LLC-cPLA$_2$ cells) expressed more cytosolic PLA$_2$ activity than the native cells, 100,000 $\times$ *g* supernatants and pellets of cellular homogenates were assayed for *in vitro* activity using 1-stearoyl-2-[1-^{14}C]arachidonyl phosphatidylcholine as a substrate. The PLA$_2$-specific activity in the cytosolic fraction of the LLC-cPLA$_2$ cells was approximately 69 ± 13 pmol/mg/min, a level that was approximately 50-fold higher than that of vector-transfected or parental LLC-PK$_1$ cells. The cytosolic activity represented approximately one-half of the total cellular activity in the LLC-cPLA$_2$ cells. This level of activity in the LLC-cPLA$_2$ cells was comparable with PLA$_2$ activities measured in cytosolic extracts of MDCK and kidney mesangial cells using the same assay system (data not shown). Thus, the PLA$_2$ activity in the LLC-cPLA$_2$ cells is in the physiological range of other kidney cell lines.

To confirm functional activity of the overexpressed cPLA$_2$, cells were labeled overnight with [^{3}H]arachidonic acid, and basal and stimulated release of [^{3}H]arachidonic acid in LLC-cPLA$_2$ and vector-transfected cells were compared. Even when not stimulated, there was a statistically significant small increase in [^{3}H]arachidonic acid release in the LLC-cPLA$_2$ cells (Fig. 1*A*). Over time more [^{3}H]arachidonic acid was released from normally growing LLC-cPLA$_2$ than LLC-PK$_1$ cells. These data are consistent with enhanced basal functional cPLA$_2$ activity in normally growing LLC-cPLA$_2$ cells. When treated with 100 nM PMA, 2 or 10 μM A23187, or PMA and A23187 together for 30 min LLC-cPLA$_2$ cells released a large amount of the radiolabeled arachidonic acid while there was no increased release in the LLC-vector cells (Fig. 1*B*) or parental LLC-PK$_1$ cells (data not shown). Thus the stimulated [^{3}H]arachidonic acid release in the LLC-cPLA$_2$ cells resulted entirely from cPLA$_2$ activation.

Having established that the cPLA$_2$-transfected cells have both more biochemical and functional PLA$_2$ activity, we then confirmed the presence of cPLA$_2$ protein by immunoblotting. Cellular proteins were separated by SDS-PAGE and detected with a polyclonal antibody raised against porcine spleen cPLA$_2$ (Fig. 2). The control cells with very low levels of PLA$_2$ activity had no detectable band. In contrast, a single 100-kDa band was seen in extracts from LLC-cPLA$_2$ cells, confirming the presence of the cPLA$_2$ protein in the transfected cells.

A stable cell line, LLC-sPLA$_2$, was created by transfection of sPLA$_2$ into LLC-PK$_1$ cells, as described under "Experimental Procedures." The specific activities of sPLA$_2$ measured in the cytosolic and membrane-associated fractions were greater than 800 and 500 nmol/mg/min, respectively. sPLA$_2$ activity in the cells transfected with vector alone or in LLC-cPLA$_2$ cells were below the measurement limit and less than 1% of the levels measured in the stable transfectants using an assay that was optimized for sPLA$_2$. This level of expression is greater than that measured by Kramer *et al.* (41) using a co-transfection expression system. Because the cDNA for sPLA$_2$ contains a signaling sequence, we expected some of it to be actively secreted from the cells. After growing LLC-sPLA$_2$ cells for 24 h,

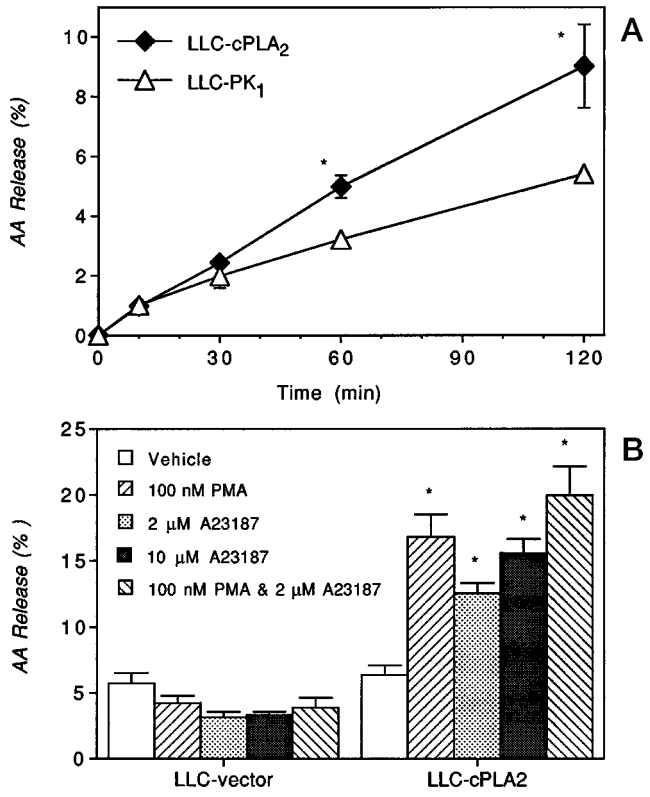

FIG. 1. **Arachidonic acid release in cPLA$_2$ expressing (LLC-cPLA$_2$) and control cells.** *A*, arachidonic acid release in nonstimulated cells. LLC-cPLA$_2$ or the parental LLC-PK$_1$ cells in DMEM were labeled with [^{3}H]arachidonic acid overnight, washed, and incubated in serum-free medium with 0.2% BSA at 37 °C. Aliquots of medium were removed at indicated times, centrifuged, and analyzed for [^{3}H]. At the end of the incubation period, cells were lysed, [^{3}H] measured, and [^{3}H] released into the medium expressed as a percent of total (medium + cellular [^{3}H]). $n = 3$. * $p < 0.05$ compared with % release from LLC-PK$_1$ cells at same time point. *B*, arachidonic acid release stimulated by PMA and A23187. Cells were treated with vehicle, 100 nM PMA, 2 or 10 μM A23187 or 100 nM PMA and 2 μM A23187 together, for 30 min at 37 °C. Percent release of [^{3}H] was determined as described in *A*. $n = 8$–12. *, $p < 0.001$ compared with vehicle-treated LLC-cPLA$_2$ or LLC-vector cells.

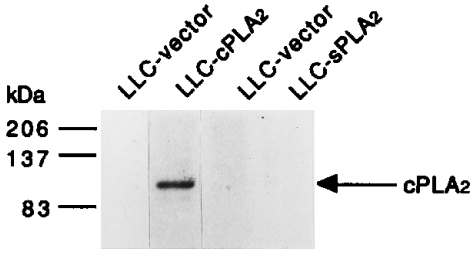

FIG. 2. **Immunoblot of extracts from LLC-cPLA$_2$, LLC-sPLA$_2$, and control LLC-vector cells.** After SDS-PAGE of 30 μg of cellular protein and transfer to a polyvinylidene difluoride membrane, the membrane was exposed to polyclonal antibody raised against porcine cPLA$_2$. The *arrow* indicates the location of the 100-kDa band corresponding to cPLA$_2$, only present in the LLC-cPLA$_2$ cells.

the measured sPLA$_2$ activity in 22-mm wells containing 1 ml of culture medium was ≈15 nmol/ml/min. This value is equivalent to the levels secreted by transfected Chinese hamster ovary cells (42) and about one-fourth to one-third the levels that are found in the inflammatory joint fluid of human subjects (43). The medium of the LLC-vector cells and the LLC-cPLA$_2$ cells did not demonstrate detectable sPLA$_2$ activity. The cPLA$_2$ activity in LLC-sPLA$_2$ cells was also assayed using [^{14}C]PC as a substrate in an assay system optimized for measurement of cPLA$_2$ activity. There was no increase in the cPLA$_2$ activity of the LLC-sPLA$_2$ cells above that of the LLC-PK$_1$ cells or the vector-transfected cells.

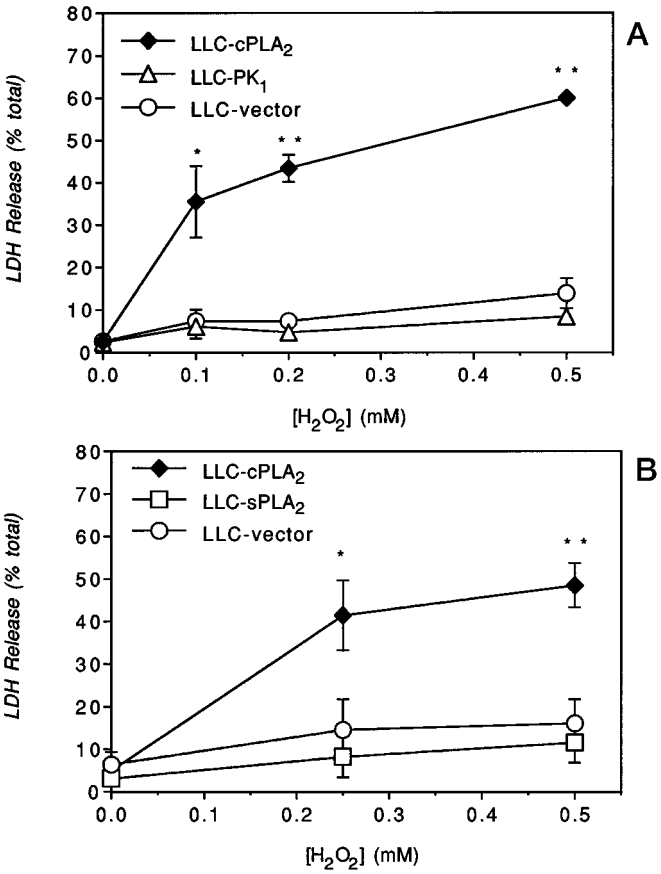

FIG. 3. **Comparison of H_2O_2 toxicity in PLA$_2$ expressing and control cell lines.** *A*, comparison of H_2O_2-induced toxicity in LLC-cPLA$_2$, LLC-PK$_1$, and LLC-vector cells. Cells were exposed to various concentrations of H_2O_2 for 3 h at 37 °C. LDH released into the medium is expressed as a percent of total LDH activity. $n \geq 6$. *, $p < 0.05$, **, $p < 0.0001$ comparing LLC-cPLA$_2$ with either control cell line. *B*, comparison of H_2O_2-induced toxicity in LLC-cPLA$_2$, LLC-sPLA$_2$, and LLC-vector cells. Cells were exposed to various concentrations of H_2O_2 for 3 h at 37 °C. LDH released into the medium is expressed as a percent of total LDH activity. $n = 4$. *, $p < 0.05$; **, $p < 0.01$ comparing LLC-cPLA$_2$ with either LLC-sPLA$_2$ or LLC-vector at equivalent H_2O_2 concentrations.

H_2O_2-induced Injury Is Enhanced in the LLC-cPLA$_2$ Cells but Not in LLC-sPLA$_2$ Cells—The effect of H_2O_2 on cell death in the cPLA$_2$ expressing cells was evaluated. A marked increase in intracellular H_2O_2 resulted from exposure to extracellular H_2O_2, as confirmed by use of a fluorescent indicator of intracellular H_2O_2 [5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate] that was introduced into cells (data not shown). As seen in Fig. 3, *A* and *B*, incubation of cells for 3 h at 37 °C in serum-free medium with H_2O_2 resulted in a dose-dependent increase in LDH release in LLC-cPLA$_2$ cells. At the same concentrations of H_2O_2, there was only minimal toxicity in the LLC-PK$_1$ or LLC-vector cells (Fig. 3*A*). For example, after 3 h in 0.5 mM H_2O_2 there was 60.0 ± 1.6% cell death in the LLC-cPLA$_2$ *versus* 13.9 ± 3.5% in the LLC-vector and 8.4 ± 0.7% in the LLC-PK$_1$. The cytotoxicity measured in the control cells is in general agreement with that found by others (44) who have studied H_2O_2-induced injury in LLC-PK$_1$ cells.

To evaluate whether this effect of cPLA$_2$ expression on H_2O_2-induced injury was a general effect due to increased cellular PLA$_2$ activity or was specific to cPLA$_2$, we examined the effect of sPLA$_2$ overexpression in the same injury model. As seen in Fig. 3*B*, incubation of LLC-sPLA$_2$ cells for 3 h at 37 °C in

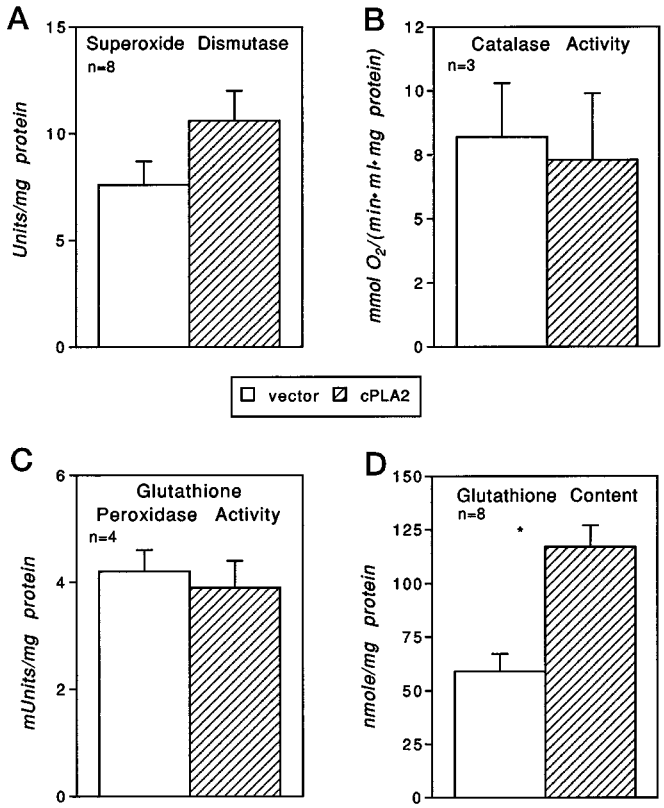

FIG. 4. **Antioxidant defenses in LLC-cPLA$_2$ and LLC-vector cells.** Activities of the antioxidant enzymes, superoxide dismutase (*A*), catalase (*B*), glutathione peroxidase (*C*), and the total glutathione content (*D*), were measured in the LLC-cPLA$_2$ and LLC-vector cells by procedures described under "Experimental Procedures." The number of replicates performed in each assay is indicated on the figure. *, $p < 0.001$ comparing LLC-cPLA$_2$ with LLC-vector cells.

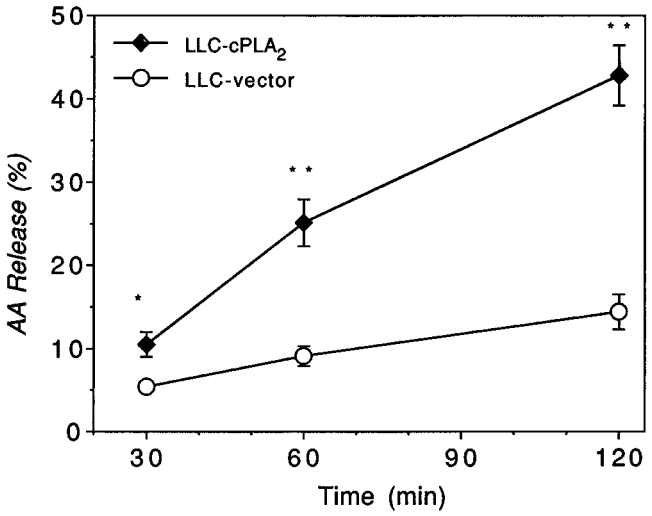

FIG. 5. **H_2O_2-stimulated arachidonic acid release.** Cells were labeled with [^{3}H]arachidonic acid, washed, and exposed to 0.5 mM H_2O_2 in DMEM with 0.2% BSA. At indicated times, aliquots of medium were removed and analyzed for [^{3}H] that was expressed as a percent of total. $n = 6$. *, $p < 0.05$; **, $p < 0.001$ comparing LLC-cPLA$_2$ with LLC-vector cells.

serum-free medium with H_2O_2 resulted in no increase in toxicity when compared with vector-transfected LLC-vector cells. This is in marked contrast to simultaneously treated LLC-cPLA$_2$ cells that released 41 ± 8.2% of total LDH upon exposure to 0.25 mM H_2O_2 and 48 ± 5.2% after exposure to 0.5 mM H_2O_2.

Antioxidant Defenses in LLC-cPLA$_2$ Cells—To evaluate the possibility that the toxicity associated with increased cPLA$_2$ expression might be due to an indirect effect on the cellular antioxidant defense system, the activities of superoxide dismutase, catalase, and glutathione peroxidase enzymes and cellular glutathione levels were evaluated in the LLC-cPLA$_2$ and LLC-vector cells. The addition of 1 mM potassium cyanide completely inhibited superoxide dismutase activity in the cells, indicating that the primary superoxide dismutase activity is the Cu-Zn form of the enzyme. As shown in Fig. 4 there were no differences in activities of superoxide dismutase, catalase, and glutathione peroxidase when comparing LLC-cPLA$_2$ to vector-transfected cells. There was, however, a significant increase in the level of glutathione in the LLC-cPLA$_2$ cells (117 ± 8 nmol/mg) compared with the LLC-vector cells (59 ± 10 nmol/mg; $p < 0.001$).

H_2O_2 Stimulates Arachidonic Acid Release in LLC-cPLA$_2$ but Not in LLC-sPLA$_2$ Cells—To confirm that functional PLA$_2$ activity is increased by H_2O_2 in the cPLA$_2$ expressing cells, [^{3}H]arachidonic acid release was measured in prelabeled cells exposed to H_2O_2 (Fig. 5). H_2O_2 (0.5 mM) stimulated significantly more [^{3}H]arachidonic acid release in the cPLA$_2$-transfected LLC-CPLA$_2$ than in the LLC-vector cells. Moreover, the [^{3}H]arachidonic acid release at 30 min (LLC-cPLA$_2$ 10.5 ± 1.5% *versus* 5.4 ± 1.1% in LLC-vector, $p = 0.02$) preceded measurable cytotoxicity (data not shown), indicating that cPLA$_2$ activation occurred prior to cell death.

The effect of 0.5 mM H_2O_2 on arachidonic acid release was also studied in LLC-sPLA$_2$ cells. In contrast to the results with LLC-cPLA$_2$ cells, there was no increase in arachidonic acid release in the LLC-sPLA$_2$ cells. The differences in response of LLC-cPLA$_2$ and LLC-sPLA$_2$ cells were also apparent when cells were exposed to the 100 nM PMA and 2 μM A23187. There was an increase in arachidonic acid release in the LLC-cPLA$_2$ cells but not in the LLC-sPLA$_2$ cells (Fig. 6). In addition, when the LLC-sPLA$_2$ cells were exposed to the ethanol vehicle, 100 nM PMA, or 2 μM A23187 alone there was no increase in arachidonic acid release (data not shown). Incubation of the LLC-sPLA$_2$ cells with 0.25, 0.5, or 1.0 mM H_2O_2 for 3 h did not result in a measurable increase in media sPLA$_2$ activity, although there was a small dose-dependent increase in cellular sPLA$_2$ activity (data not shown).

Menadione-induced Injury—To establish whether the susceptibility to injury can be generalized to other oxidants and a different method of evaluating cell injury, we exposed cPLA$_2$ expressing cells in suspension to menadione, a superoxide anion generator (45), and evaluated toxicity by trypan blue staining. After exposure to 50 μM menadione for 90 min at 37 °C, we found significant irreversible injury in the LLC-cPLA$_2$ (28.8 ± 2.7% trypan blue-positive cells *versus* vehicle-treated, 9.0 ± 0.6%) but not in the LLC-vector (13.0 ± 2.4% *versus* vehicle-treated, 10.4 ± 0.9%, $p > 0.5$).

Exogenous Arachidonic Acid Does Not Enhance H_2O_2 Toxicity—Arachidonic acid and its metabolites have been shown to be injurious in several situations. However, neither normal nor injured LLC-PK$_1$ cells have been reported to have measurable cyclooxygenase (46) or lipoxygenase (47) activities. This suggests that arachidonic acid metabolites are not present or exist only in low concentrations in the native cell line. Nevertheless, it is possible that the enhanced toxicity of H_2O_2 in LLC-cPLA$_2$ results from the toxic effect of liberated arachidonic acid and/or its metabolites, rather than a direct effect of PLA$_2$ on cell membranes or liberated lysophospholipids. To test that possibility, we exposed LLC-cPLA$_2$ cells to 150 μM arachidonic acid in the presence or absence of simultaneous exposure to H_2O_2. There was no potentiation of cell death in cells treated with arachidonic acid whether the cells were simultaneously ex-

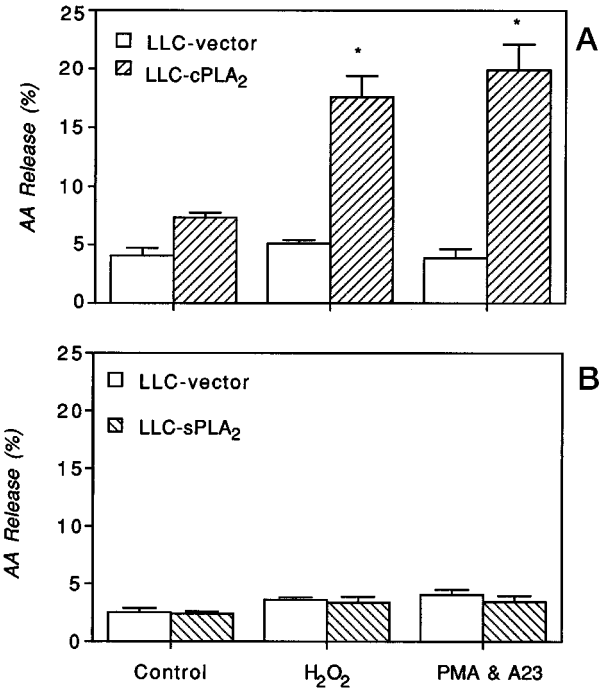

FIG. 6. **Effects of H_2O_2 and PMA and ionophore on arachidonic acid release from LLC-cPLA$_2$ (*A*), LLC-sPLA$_2$ (*B*), and LLC-vector cells.** Cells were prelabeled with [^{3}H]arachidonic acid, and release of [^{3}H]arachidonic acid into the medium was measured after 30 min of treatment with vehicle, 0.5 mM H_2O_2, or 100 nM PMA and 2 μM A23187. % release was then calculated by measuring total cellular [^{3}H]arachidonic acid. $n = 9$ in *A* and 6 in *B*. *, $p < 0.001$ compared with % arachidonic acid release in LLC-vector cells.

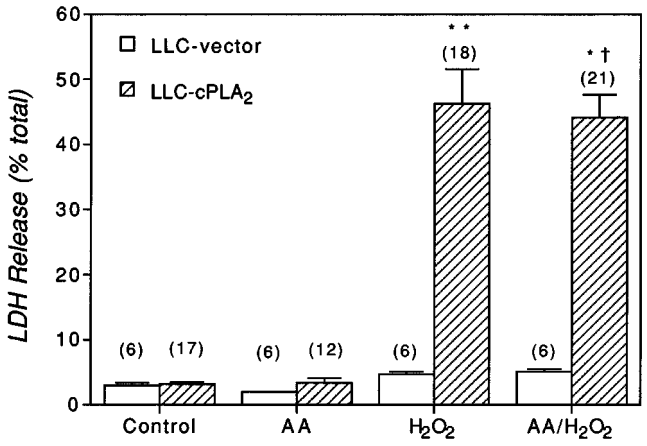

FIG. 7. **Arachidonic acid does not potentiate H_2O_2 toxicity in LLC-cPLA$_2$ or LLC-vector cells.** LLC-vector and LLC-cPLA$_2$ cells were treated with 150 μM arachidonic acid in the presence or absence of 1.0 mM H_2O_2. The arachidonic acid was added 2 min after H_2O_2 in the arachidonic acid/H_2O_2 group. LDH release was measured after 3 h of incubation. *, $p < 0.01$; **, $p < 0.005$ when compared with LLC-vector cells. †, not significantly different from H_2O_2-treated LLC-cPLA$_2$ cells.

posed to H_2O_2 or vehicle (Fig. 7).

BAPTA, but Not Glycine, Protects Against H_2O_2 Toxicity—Since the activity of cPLA$_2$ is calcium-dependent, we predicted that the treatment of LLC-cPLA$_2$ cells with a chelator of intracellular Ca^{2+} should prevent H_2O_2-induced injury. Preincubating LLC-cPLA$_2$ cells for 30 min with 100 μM BAPTA/AM in nominally Ca^{2+}-free PBS resulted in complete protection from H_2O_2 toxicity (% LDH release, 6.5 ± 2.1% in untreated cells; 65.9 ± 5.8% in vehicle plus H_2O_2; 5.6 ± 1.6% in BAPTA/AM 100 μM plus H_2O_2; $n = 9$, Table I). Introduction of BAPTA into the cells also blocked the increase in arachidonic acid release seen with H_2O_2 treatment (Table I). Addition of 2.3 mM EGTA to the medium in which the cells are incubated, for 30 min prior to H_2O_2 exposure (which reduced the extracellular [Ca^{2+}] to less than 50 nM), resulted in a 26% reduction in LDH release ($n = 8$, $p < 0.001$, data not shown).

Glycine has been shown to protect against hypoxic injury in renal tubules (48) and calcium ionophore-induced injury in cultured MDCK and LLC-PK$_1$ cells (32). The addition of 2 mM glycine provided no protection against H_2O_2-induced injury in the LLC-cPLA$_2$ cells, and glycine had no effect on arachidonic acid release from vehicle- or H_2O_2-treated LLC-cPLA$_2$ cells (Table I). In addition glycine did not provide protection from H_2O_2 toxicity to cells incubated in a nominally calcium-free buffer (data not shown).

Calcium Ionophore Toxicity—To test whether activation of cPLA$_2$ by calcium ionophore is sufficient to cause increased cell death, LLC-cPLA$_2$, LLC-PK$_1$, and LLC-vector cells were exposed to 1, 5, or 10 μM A23187 for 3 h and toxicity monitored (Fig. 8). At base-line level, the LLC-cPLA$_2$ cells had slightly higher LDH release than the other two cell lines. Despite being able to stimulate arachidonic acid release in LLC-cPLA$_2$ cells, 1 and 5 μM A23187 caused no increased cell injury. At 10 μM, there was increased cell death in each cell line, but the absolute

TABLE I
Effects of BAPTA or glycine treatment on H_2O_2-induced cytotoxicity and arachidonic acid release

LDH release was measured in LLC-cPLA$_2$ cells preincubated with 100 μM BAPTA/AM or dimethyl sulfoxide vehicle in calcium-free PBS or with 2 mM glycine or vehicle in DMEM for 30 min. The pretreated cells were exposed to 0.2 or 0.5 mM H_2O_2 for 3 h. [^{3}H]Arachidonic acid release was determined after a 30-min exposure to H_2O_2. All values are means ± S.E. of three experiments, each performed in triplicate.

Agents	LDH release	Arachidonic acid release
	% control	*% control*
Performed in PBS		
Vehicle	6.5 ± 2.1	13.0 ± 0.4
Vehicle + 0.2 mM H_2O_2	66.0 ± 5.8	19.4 ± 1.9
BAPTA + 0.2 mM H_2O_2	5.6 ± 1.6[a]	13.0 ± 1.4[a]
Performed in DMEM		
Vehicle	3.0 ± 0.2	7.3 ± 0.4
Vehicle + 0.5 mM H_2O_2	33.1 ± 1.8	17.6 ± 1.8
Glycine + 0.5 mM mM H_2O_2	28.1 ± 1.6 NS	19.8 ± 2.2 NS[b]

[a] $p < 0.01$, compared with vehicle + H_2O_2.
[b] NS, not significantly different from vehicle + H_2O_2 treatment.

increase in LDH release, over control values, in the LLC-cPLA$_2$ cells was equivalent to that of the LLC-vector cell. Thus, while cPLA$_2$ activation appears to be necessary for the enhanced oxidant injury seen in LLC-cPLA$_2$ cells, activation with calcium ionophore does not appear to be sufficient to cause toxicity by itself.

Mercuric Chloride Toxicity—Treatment of LLC-PK$_1$ cells with mercuric chloride results in the rapid accumulation of arachidonic acid and lysophospholipids and irreversible injury, leading to the suggestion that phospholipase activity may play a part in the pathogenesis of mercuric chloride toxicity (47). If this were the case, and if the PLA$_2$ involved were cPLA$_2$, then LLC-cPLA$_2$ cells would have enhanced toxicity to mercuric chloride. To investigate this possibility, LLC-cPLA$_2$ and LLC-PK$_1$ cells were exposed to 1 or 10 μg/ml mercuric chloride for 3 h and cell death measured by trypan blue uptake. No significant differences in mercuric chloride-induced toxicity were found between LLC-cPLA$_2$ and either LLC-PK$_1$ or vector-transfected cells (data not shown). Thus, cPLA$_2$ does not appear to be an important mediator of mercuric chloride injury. Furthermore, the fact that LLC-cPLA$_2$ cells are not more susceptible to A23187 or mercuric chloride toxicity suggests that

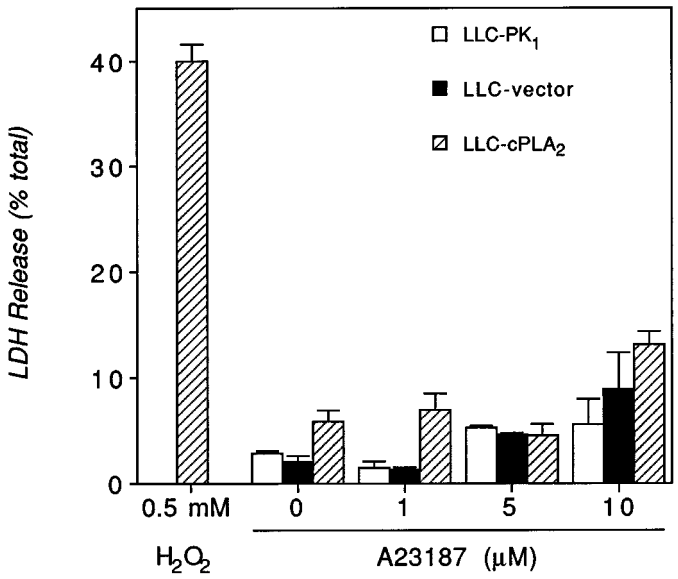

FIG. 8. **Toxicity of the calcium ionophore A23187 on LLC-cPLA$_2$ cells.** LLC-cPLA$_2$, LLC-vector, and LLC-PK$_1$ cells were treated with 1, 5, or 10 μM A23187 or vehicle for 3 h and LDH release measured. n = 3–10.

cPLA$_2$, while enhancing oxidant injury, does not potentiate injury to all toxic agents.

DISCUSSION

Changes in cellular phospholipid metabolism are found in response to various forms of cell insults and likely play a pathogenic role in cytotoxicity (8, 49–51). PLA$_2$ activation results not only in the degradation of membrane phospholipids but also the accumulation of unsaturated free fatty acids and lysophospholipids which by themselves can be injurious. Determining the exact role PLA$_2$ plays in pathogenesis is complicated by the interrelated pathways leading to cell injury that are induced by pathophysiological states, including increased intracellular [Ca^{2+}], ATP depletion, and the generation of reactive oxygen species (2). Furthermore, an evaluation of the role of PLA$_2$ in cell injury is also complicated by the fact that multiple forms of mammalian PLA$_2$ exist and many cells have more than one form. The various forms of the enzyme have many different characteristics, including calcium sensitivity, pH optima, and substrate specificity of the polar head group, the type of bond at the *sn*-1 position, and the fatty acid at the *sn*-2 position of the phospholipid (24, 25). An understanding of the role of this class of enzymes will depend on an approach designed to examine the role of each individual isozyme. The development of cell lines expressing predominantly one type of PLA$_2$ offers an opportunity to examine what role, if any, a particular form of PLA$_2$ might play in different forms of injury.

The cytosolic PLA$_2$ (cPLA$_2$) demonstrates specificity for arachidonic acid at the *sn*-2 position of phospholipids, translocates to membranes at calcium concentrations found in stimulated cells, and is activated by phosphorylation in response to hormonal stimulation (31, 36, 42, 52). An essential role for cPLA$_2$ has been proposed in cytotoxicity and apoptosis resulting from tumor necrosis factor (53, 54) and ischemia in kidney (8) and brain (20). In the current study, we have demonstrated that expression of cPLA$_2$, in cells with nonmeasurable baseline levels, results in enhanced susceptibility to H_2O_2-induced injury. Oxidant injury in LLC-PK$_1$ cells has been examined by others (44, 55) but with little attention paid to the possible role of PLA$_2$. Oxidative stress may have a direct effect on the function of cPLA$_2$ enhancing the cytotoxicity of this enzyme. Lipid peroxidation has been reported to stimulate PLA$_2$ activity, perhaps by providing a preferable substrate for hydrolysis (56–58). Additionally, exposure of LLC-PK$_1$ cells to 1 mM H_2O_2 for as little as 10 min has been shown to raise intracellular free [Ca^{2+}] by more than 100 nM (59), and the increase in [Ca^{2+}] is likely to result in translocation of cPLA$_2$ to membranes where its substrates reside (26, 31, 36). Taken together, peroxidation of membrane phospholipids and rising intracellular [Ca^{2+}] can account for cPLA$_2$ activation in response to H_2O_2 exposure.

The enhanced susceptibility of LLC-cPLA$_2$ cells to an oxidant injury was not due to alterations in the antioxidant defense system. Both increases and decreases in the cellular levels of superoxide dismutase have been implicated in models of cell death (16, 60–63). The amount of superoxide dismutase activity in LLC-cPLA$_2$ cells was slightly higher than in the vector-transfected cells. While this difference was not statistically significant, we considered whether such a small difference might be physiologically relevant. Increased superoxide dismutase activity has been demonstrated to cause cytotoxicity; however, toxicity was dependent upon substantial increases in the superoxide dismutase activity (16, 63). It has been reported that hydroperoxides released by PLA$_2$ can be detoxified by glutathione peroxidase (64). Interestingly, the total cellular glutathione content was increased in LLC-cPLA$_2$ cells as compared with levels in LLC-vector cells. Other investigators (65, 66) have demonstrated that exposure to nonlethal oxidant stress or prostaglandin A$_2$ cause elevations of glutathione levels in cultured cells. This increase has been related to increased transcription of γ-glutamylcysteine synthetase that is the rate-limiting enzyme in glutathione synthesis (66). It is possible that the basal increase in arachidonic acid release of the LLC-cPLA$_2$ cells (Fig. 1*A*) may increase glutathione levels through a similar mechanism that is dependent upon arachidonic acid or one of its metabolites. Other mechanisms such as alterations in the transmembrane processing of glutathione could also account for the observed increase (67). Ballmaier and Epe (68) have shown that increased glutathione levels in combination with H_2O_2 do not result in significant synergistic DNA damage. It is unlikely that the increase in glutathione accounts for the enhanced toxicity of H_2O_2 in the LLC-cPLA$_2$ cells.

The enhanced toxicity of H_2O_2 is not a generalized response to increased cellular PLA$_2$ activity because cells transfected with sPLA$_2$ were not more susceptible to injury when exposed to H_2O_2. In contrast to cPLA$_2$, sPLA$_2$ is not specific for arachidonic acid at the *sn*-2 position of fatty acids and requires higher calcium concentrations for its activity. The level of sPLA$_2$ protein in the medium of LLC-sPLA$_2$ cells approached that found in inflammatory joint fluids, and the total cellular level was greater than 100 times the amount in vector-transfected cells. The lack of cell toxicity associated with markedly elevated levels of sPLA$_2$ may be due to nonaccessibility of sPLA$_2$ to cellular membranes since group II PLA$_2$ may be packaged within vesicles, isolating it from the remainder of the cell. Alternatively, the membranes of the cell may not be susceptible to enzymatic attack by group II PLA$_2$. It has been proposed that phospholipid rearrangement, by exposing preferential lipid substrates for the enzyme, may potentiate group II PLA$_2$ activity (43, 69). While A23187 has been proposed to enhance sPLA$_2$ activity in other cells due to ionophore-induced changes in membrane asymmetry (70), the activity of sPLA$_2$ was not increased in LLC-sPLA$_2$ cells in response to H_2O_2, A23187, or the combination of A23187 and PMA in our studies. Lin *et al.* (42) have also shown that sPLA$_2$-transfected Chinese hamster ovary cells were unresponsive to ionophore and hormone stimulation.

The Ca^{2+}-induced activation of cPLA$_2$ alone does not appear to be toxic to LLC-cPLA$_2$ cells, because doses of A23187 that

stimulated arachidonic acid release produced no increase in toxicity. The absence of cell death in the presence of cPLA$_2$ activation by nontoxic doses of ionophore is not surprising since many physiological stimuli increase intracellular free [Ca^{2+}] and activate cPLA$_2$ without pathophysiological consequences. The activation of the enzyme in response to H_2O_2 may result in toxicity because, in addition to increases in intracellular free [Ca^{2+}], there are concomitant changes in plasma or intracellular membrane susceptibility to cPLA$_2$. The intracellular site of cPLA$_2$ activity may be altered by exogenous stimuli (71).[2] It is possible that H_2O_2 directs cPLA$_2$ activity to a cellular location, such as the nucleus, where it produces increased toxicity. The protection against H_2O_2 toxicity afforded by BAPTA is consistent with the importance of intracellular Ca^{2+} as a co-mediator with H_2O_2 of cell death in our system. Chelation of intracellular Ca^{2+}, however, will also affect other Ca^{2+}-dependent enzymes such as proteases (72–74), and this could be an important factor in limiting the toxicity of H_2O_2. The limited protective effect of extracellular Ca^{2+} depletion might be expected as the rise in intracellular [Ca^{2+}] after exposure to H_2O_2 is only minimally dependent on the presence of extracellular Ca^{2+} (44). Furthermore, cPLA$_2$ requires only small increases in intracellular [Ca^{2+}] to translocate to membrane phospholipids (75).

In these studies we considered the possibility that the generation of arachidonic acid and/or its metabolites in the setting of oxidant stress might lead to metabolites that mediate the H_2O_2 toxicity. When arachidonic acid was added to H_2O_2-treated LLC-cPLA$_2$ cells, however, no enhancement of injury was found. This is in general agreement with the observations of others (46, 47) who found LLC-PK$_1$ cells to produce few, if any, cyclooxygenase and lipoxygenase products and to be relatively resistant to the toxicity of exogenous lipids (47). These data suggest that the generation of arachidonic acid and/or its metabolites does not play an important role in the increased toxicity of H_2O_2 in LLC-cPLA$_2$ cells. It is possible, however, that exogenously administered arachidonic acid may not mimic the effects of endogenously generated arachidonic acid. Zager and colleagues (23) have found that large amounts of group II PLA$_2$ or arachidonic acid added exogenously to isolated rat proximal tubules limited injury in an *in vitro* model of hypoxia/reoxygenation. These authors suggested that protection was due to feedback inhibition of endogenous cPLA$_2$ by arachidonic acid or its metabolites.

Our studies reveal that pretreatment of cells with glycine, which has been shown to be protective against hypoxic ATP depletion and calcium ionophore-induced injury in isolated kidney tubules and cultured LLC-PK$_1$ and MDCK cells (32, 48), confers no protection against H_2O_2-induced toxicity. In a model, in which exogenous group II PLA$_2$ was injurious to proximal tubule cells, Wetzels and co-workers (76) found that glycine was not protective. By contrast, this group found that glycine protected against the toxicity of exogenous arachidonic acid and concluded that the cell injury caused by PLA$_2$ is related to membrane degradation and not the liberated fatty acids. Venkatachalam *et al.* (77) reported that glycine had no effect on free fatty acid release although it protected MDCK cells treated with calcium ionophore and an uncoupler of oxidative phosphorylation. Thus, the protective effects of glycine on renal tubule cells is independent of PLA$_2$.

Our determination that neither A23187 nor mercuric chloride caused more cell death in the LLC-cPLA$_2$ cells suggests that there is some specificity to the synergism that exists between cPLA$_2$ activity and oxidant stress that results in cell injury. Another demonstration of the specificity of the synergy between cPLA$_2$ and H_2O_2 is the fact that sPLA$_2$ overexpressing cells show no enhanced susceptibility to H_2O_2-induced injury even though the amount of group II PLA$_2$ protein in these cells is very high.

The exact nature of the interaction between H_2O_2 and cPLA$_2$ that results in injury remains to be determined, but our findings support the following hypothesis. The treatment of cells with H_2O_2 leads to lipid peroxidation and rising intracellular [Ca^{2+}], both of which could increase cPLA$_2$ activity. The enhanced release of peroxidized fatty acids and lysophospholipids by the highly expressed cPLA$_2$ may overwhelm the normal adaptive responses to oxidant stress, including the reduction and reacylation of the liberated fatty acids. The loss of membrane integrity would lead to further alterations in [Ca^{2+}] homeostasis and subsequent cell death.

Alternatively, the enhanced cytotoxicity of H_2O_2 in LLC-cPLA$_2$ cells may arise from a downstream effect of cPLA$_2$ expression. Nuclear localization of cPLA$_2$ may have important implications for its role in intracellular signaling and gene expression. We have found an increase in the glutathione levels of cells expressing cPLA$_2$, and it is likely that the expression of other cellular proteins is also altered. Further exploration of such changes may elucidate the mechanisms of cytotoxicity associated with cPLA$_2$.

In conclusion, cell lines have been created to investigate the roles of two forms of PLA$_2$ in cellular injury. Expression of cPLA$_2$ in LLC-PK$_1$ cells, to levels found normally in many other cell types, confers enhanced susceptibility to H_2O_2-mediated injury. By contrast, cells that overexpress sPLA$_2$ show no increased susceptibility to H_2O_2-mediated injury. Increases in intracellular calcium concentration are necessary but not sufficient to produce enhanced toxicity of H_2O_2 in cells expressing cPLA$_2$. Glycine did not protect these cells against oxidant injury. Thus, we propose that cPLA$_2$, which is expressed in many cells, but not a group II PLA$_2$, is an important endogenous mediator of oxidant-induced cell injury.

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