Interleukin 1α Causes Rapid Activation of Cytosolic Phospholipase A2 by Phosphorylation in Rat Mesangial Cells

Joseph Gronich, Martha Konieczkowski, Michael H. Gelb,* Raphael A. Nemenoff,† and John R. Sedor
Departments of Medicine, and Physiology and Biophysics, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44109; * Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195; and † Department of Medicine, University of Colorado Health Sciences Center, Denver, Colorado 80262

Abstract

We have shown previously that interleukin I (IL-1) stimulates eicosanoid production in glomerular mesangial cells (MC) by de novo synthesis of a 14-kD, group II phospholipase A2 (PLA2). IL-1-stimulated prostaglandin E2 synthesis precedes expression of this enzyme, suggesting that another PLA2 isozyme must be more rapidly activated. In the presence but not absence of calcium ionophore, [3H]arachidonate release is increased significantly as early as 5 min after addition of IL-1, and IL-1 concurrently stimulates a Ca2+-dependent phospholipase activity, which was characterized as the cytosolic form of PLA2 (cPLA2). IL-1 does not alter either cPLA2 mRNA expression or mass in serum-stimulated MC, suggesting that cPLA2 activity is increased by a posttranslational modification. IL-1 treatment for 30 min doubles 32P incorporation into immunoprecipitable cPLA2 protein, concordant with the increase in enzyme activity. Immunoblot analysis of extracts derived from IL-1–treated (30 min) cells demonstrates a decreased mobility of cPLA2, and treatment of MC lysates with acid phosphatase significantly reduces cytokine-activated cPLA2 activity, further indicating that IL-1 stimulates phosphorylation of the enzyme. IL-1 treatment (24 h) of serum-deprived MC doubled cPLA2 mRNA, protein, and activity. In summary, IL-1 increases cPLA2 activity in a biphasic, time-dependent manner both by posttranslational modification and de novo synthesis. We consider cPLA2 activation a key step in IL-1–stimulated synthesis of pro-inflammatory, lipid mediators, and an integral event in the phenotypic responses induced in target cells by this cytokine. (J. Clin. Invest. 1994. 93:1224–1233.) Key words: arachidonic acid · cellular signaling · cytokine · eicosanoid metabolism · inflammation

Introduction

IL-1 is a potent pro-inflammatory cytokine that activates functional responses and biochemical processes in target cells (1). The release of arachidonic acid–derived lipid mediators is a characteristic of chronic and acute inflammation, and IL-1 stimulates eicosanoid synthesis in vitro (1). The molecular mechanisms of IL-1–stimulated arachidonate metabolism have not been defined completely, but receptor-activated, phospholipase A2 (PLA2)1–mediated release of arachidonic acid from the sn-2 position of membrane phospholipids is rate-limiting in eicosanoid biosynthesis (2).

Mammalian PLA2s, until recently, have been classified into two groups (I and II) based on position of cysteine pairs and sequence similarities to families of 14-kD PLA2 isozymes secreted in snake venoms (3). Mammalian pancreatic PLA2 is a group I enzyme. Mammalian group II enzymes are synthesized in many tissues and abundantly secreted into inflammatory exudates. Both groups I and II secretory PLA2s (sPLA2) require millimolar calcium concentrations for half-maximal enzyme activity and do not demonstrate preference for the fatty acid present in the sn-2 position of phospholipids. Recently a 97-kD cytosolic form of PLA2 (cPLA2) has been identified in glomerular mesangial cells and the human monocyte cell line U937 (4–6). This enzyme translocates to membrane vesicles in response to nanomolar changes in free Ca2+ concentration and selectively hydrolyzes arachidonyl-phospholipids in membrane vesicles (7). The primary structure of cPLA2 lacks any identity with either cloned sPLA2, and its biochemical characteristics suggest that this form of PLA2 regulates intracellular arachidonic acid release (4–7).

Enhanced prostaglandin synthesis characterizes glomerular inflammation, and recent evidence suggests that IL-1 is important in the pathogenesis of glomerular injury (8, 9). Mesangial cells are the most abundant source of prostaglandins within the glomerulus, and in culture, express a phenotype that mimics their characteristics within an inflamed glomerulus. We believe IL-1–stimulated mesangial cells to be an appropriate in vitro model system to define the molecular basis for the enhanced arachidonate availability that characterizes glomerular injury.

We have demonstrated previously that IL-1 specifically stimulates mesangial cells to synthesize a group II (nonpancreatic type) sPLA2 (10). IL-1–dependent induction of type II sPLA2 mRNA expression increased coordinately with PLA2 activity and PGE2 synthesis. However, three observations reported in this study suggested that a PLA2 distinct from this sPLA2 isoform also must be activated by IL-1 stimulation. First, PGE2 synthesis occurred before 6–10 h of IL-1 stimulation, when sPLA2 mRNA is first detected. Second, IL-1 did not

1. Abbreviations used in this paper: AA-COOCH3, the trifluoromethyl ketone analogue of arachidonic acid which inhibits cPLA2 activity; AA-COCH3, an arachidonate analogue devoid of inhibitory activity for cPLA2; MAP, mitogen-activated protein; PLA2, phospholipase A2; similarly, cPLA2 and sPLA2, cytosolic form of and 14-kD secretory PLA2, respectively; PLAP, PLA2-activating protein.

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Address correspondence to Dr. John R. Sedor, Department of Medicine, MetroHealth Medical Center, 2500 MetroHealth Drive/Office A1112, Cleveland, OH 44109-1998.

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increase basal [3H]arachidonic acid release but did potentiate vasopressin-stimulated arachidonate release. Because cPLA₂ has been shown to be regulated by vasopressin (4), we reasoned that this PLA₂ may be primed by IL-1 for enhanced, agonist-stimulated arachidonic acid release. Third, although phosphatidylcholine is hydrolyzed poorly by group II sPLA₂, IL-1 increased hydrolysis of arachidonic acid from phosphatidylcholine, a finding consistent with enhanced cPLA₂ activity. Taken together, these data suggest that IL-1 directly or indirectly activates cPLA₂. We now report that IL-1 rapidly increases cPLA₂ activity by changing the phosphorylation state of the enzyme, and in longer incubations, can increase catalytic activity by inducing cPLA₂. cPLA₂ activation and enhanced arachidonate availability is integral to the glomerular synthesis of pro-inflammatory eicosanoids stimulated by IL-1.

Methods

Materials. Recombinant human IL-1α (IL-1, 3 × 10⁶ U/mg) was kindly provided by Dr. Peter Lomedico (Hoffmann-LaRoche, Nutley NJ). The endotoxin content and specific activity have been previously published (10). L-α-1-palmitoyl, 2-arachidonyl, [1-¹⁴C]arachidonoylphosphatidylethanolamine (60 Ci/mmol), and arachidonic acid (5, 6, 8, 9, 11, 12, 14, 15, ³⁵H(N)), 100 Ci/mmol) were purchased from DuPont New England Nuclear (Boston, MA). L-α-1-stearoyl, 2-arachidonyl, [1-¹⁴C]arachidonoyl-phosphatidylcholine (56 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). ⁳²P, orthophosphate (carrier free), trans ⁳²S-label (L-methionine, [⁳²S]), 1,163 Ci/mmol), phosphate-free RPMI 1640 and methionine, cysteine-free RPMI 1640 medium were obtained from ICN Biomedicals, Inc. (Irvine, CA).

Cell culture. Well-characterized mesangial cells were grown from collagenase-treated rat glomeruli in RPMI 1640 medium containing 8.5% FBS and 8.5% calf serum, 15 mM Heps, 100 U/ml penicillin, 100 μg/ml streptomycin and fungizone, 5 μg/ml of insulin and transferrin, and 5 ng/ml of sodium selenite as previously described (10). The cells were used in passages 3–8, and as indicated in the specific methods or in the results were either maintained in 17% serum, acutely serum-deprived (2 h) or were held for 24–48 h in 0.5% FBS. [³⁵H]Arachidonate release. Basal and stimulated arachidonate release was determined as previously described (10), with some modifications. Cells in six-well clusters were labeled for 20 h with 0.5 μCi/ml [³⁵H]arachidonic acid in RPMI 1640 medium with 17% serum. The labeling media was aspirated and the cells washed and serum-depleted for 2 h at 37°C in Heps (10 mM)-buffered RPMI (Heps-RPMI) containing 0.1% BSA. The experiment was initiated by the addition of vehicle or IL-1 for 1, 4, or 20 min before the addition of the Ca²⁺ ionophore, A23187 (0.25 μM) (Calbiochem Corp., San Diego, CA). This A23187 concentration was chosen to cause only a minimal change in [³⁵H]arachidonate release (Fig. 1) and to mimic a receptor-mediated increase in cytosolic Ca²⁺ (M. B. Ganz, personal communication). Ionophore was added for an additional 1 min after the cells were incubated with IL-1 for 1 and 4 min, and for 10 min after the cells had been exposed to IL-1 for 20 min for total incubation times of 2, 5, and 30 min, respectively. As positive controls, cells were also incubated with PMA for 10 min followed by an additional 10 min in the presence of ionophore. In some experiments, the cells were preincubated for 2 h with a control or cPLA₂-inhibitory arachidonate acid analogues before stimulation with IL-1 or PMA (11). All reactions were terminated by removal of the incubation media and the addition of 0.4 ml of cold (4°C) methanol containing 10 μg unlabeled arachidonic acid to the monolayer. The cells were scraped, and this suspension along with an additional 0.4 ml methanol rinse was combined with the incubation supernatants and acidified with formic acid (0.2%, vol/vol). Chloroform/methanol (1:1.2, vol/vol, 0.6 ml) was added to the extraction tube followed by the addition of 0.6 ml of chloroform. After separating the organic and aqueous phases by centrifugation, the organic phase was dried under N₂ and resolubilized in ethanol. Free arachidonate was separated from cellular phospholipids using TLC. [³⁵H]Arachidonate acid release was expressed as a percentage of total [³⁵H]arachidonate incorporated into the mesangial cell monolayer (determined by total TLC plate radioactivity).

Cell-free extracts and PLA₂ assay. At the time of the experiment, subconfluent cells were serum-restricted by replacing growth medium with experiments of triplicate wells (n = number of separate experiments; *P < 0.05, **P < 0.025 compared to the control value). In the absence and presence of IL-1, ionophore-stimulated [³⁵H]arachidonate release at 2 min was 4.4 ± 0.6% and 5.1 ± 0.8% release (n = 4, ionophore- vs. ionophore- and IL-1-stimulated, respectively, P = NS); at 5 min was 4.5 ± 0.4% vs. 6.9 ± 0.9% release (n = 3, ionophore- vs. ionophore- and IL-1-stimulated, respectively, P < 0.01); at 30 min was 5.3 ± 0.2% and 8.8 ± 0.7% release (n = 3, ionophore- vs. ionophore- and IL-1-stimulated respectively, P < 0.01). After 10 min, ionophore-stimulated [³⁵H]arachidonate release was 5.3 ± 0.2% vs. 11.0 ± 2.9% in the absence and presence of PMA, respectively (n = 3, P < 0.005).

Figure 1. IL-1 rapidly stimulates [³⁵H]-arachidonate release. Subconfluent rat mesangial cells were labeled with [³⁵H]-arachidonate acid (0.5 μCi/well) for 20 h, washed with RPMI media containing 0.1% BSA, and incubated in this media for an additional 2 h. The cells were then stimulated for the indicated times with vehicle alone (control), IL-1 (50 ng/ml), or PMA (300 nM). Cells were exposed to IL-1 for 1 and 4 min before the addition of A23187 (0.25 μM) for an additional 1 min. In the longest incubations, mesangial cells were incubated for 10 and 20 min with PMA and IL-1, respectively. A23187 was subsequently added for 10 min. The times in the figure indicate the total exposure time to IL-1 or PMA in the absence and the presence of ionophore. [³⁵H]Arachidonate in cells and supernatants was quantified by TLC after chloroform-methanol extraction. Results are the means±SEM for triplicate
with Hepes-RPMI for 2 h before incubation with IL-1, 12-tetradecanoyl phorbol myristate 13-acetate (Calbiochem Corp.) or vehicle-control at the indicated doses. These stimuli were added directly to the preincubation medium, and the cells were incubated at 37°C in the presence of 5% CO₂, 95% air for the indicated times. In some experiments, cells were treated with heated IL-1 (90°C, 60 min) or native IL-1 in the presence of polymyxin B (10 μg/ml, Sigma Chemical Co., St. Louis, MO) to determine if LPS contaminating the reagents accounted for the effects of the cytokine. Treatment of cells with polymyxin B alone did not alter cPLA₂ activity in cell-free extracts (not shown). Three 100-mm dishes of cells were used for each condition to assay PLA₂ activity in 200,000 g supernatants of cell lysates as previously described (4, 10). Briefly after the incubation period, the media was removed. The cells were washed and scraped at 4°C into a buffer containing 250 mM sucrose, 50 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM EGTA, and the protease inhibitors, pepstatin A (1 μg/ml), leupeptin (1 μg/ml), and PMSF (0.1 mM). The cells were homogenized using a Dounce homogenizer (25 strokes) and spun at 500 g for 5 min to remove the nuclei. The homogenate was centrifuged at 200,000 g for 1 h at 4°C using a model Ti-70.1 rotor (Beckman Instruments, Inc., Fullerton, CA). Protein content in the supernatant was determined (protein assay, Bio-Rad Laboratories, Richmond, CA). Samples from each condition were matched for protein concentration by appropriate dilution and assayed for PLA₂ activity. Unless otherwise indicated, [1-14C]arachidonoylphosphatidylcholine was used as substrate (final concentration, 15 μM). The assay was initiated by adding mesangial cell extract containing 1.5–7 μg of protein to an assay buffer containing 5 mM CaCl₂ and 50 mM Hepes, pH 7.5 (final concentrations). In some experiments, control and cPLA₂-inhibitory arachidonic acid analogues (11), in the indicated concentrations, were added for 5 min at 37°C to the cellular lysates in assay buffer. The assay then was initiated by the addition of [14C]phosphatidylcholine. Reactions proceeded for 30 min at 37°C and were terminated by addition of 40 μl of 2% acetic acid in ethanol containing 10 μg of unlabeled arachidonic acid. The reaction mixtures and appropriate standards were spotted onto heat-activated LK 5 DF silica gel TLC plates (Whatman Inc., Clifton, NJ) and developed with ethyl acetate/isooctane/acetic acid/water (55:75:8:100). Lipids were visualized by I₂ staining. Free arachidonate was scraped and quantified by liquid scintillation counting. Results were expressed as picomoles of arachidonate released per minute per milligram of protein.

Extract fractionation. As described (4, 12), protein-matched extracts from control, IL-1- and PMA-stimulated cells were applied to a prepacked 5 × 50-mm Mono-Q HR 5/5 anion exchange column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) preequilibrated with 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, and 50 mM Hepes, pH 7.5. The column was washed with equilibration buffer. PLA₂ activity was eluted with a linear salt gradient (0.15–1.0 M NaCl). 0.5-ml fractions were collected and assayed for PLA₂ activity.

Measurement of cPLA₂ mRNA levels. IL-1-induced changes in cPLA₂ mRNA levels were assessed by Northern analysis of total cellular RNA (20 μg) as described (13) with the following modifications. A gel-purified cDNA fragment which encoded the 2.9 kb human cytosolic PLA₂ (7, kindly provided by Dr. James D. Clark, Genetics Institute, Inc., Cambridge, MA), was labeled with [α-32P]dCTP (Dupont/NEN, Boston, MA) by nick translation. Filters were hybridized with 32P-labeled cPLA₂ probe overnight at 42°C in 5× SSPE (1× SSPE: 180 mM NaCl, 10 mM Na₂HPO₄, 7MHO, and 1 mM EDTA), 50% formamide, 5× Denhardt’s solution, 1% SDS, 10% dextran sulfate, and 200 μg/ml denatured salmon sperm DNA. The blot was subsequently washed with 2× SSPE at room temperature for 30 min; 2× SSPE, 2% SDS at 30°C for 30 min and a final wash in 0.1× SSPE, 1% SDS at 52°C. After autoradiography for 15 h, bound probes were stripped from the blots by boiling and the filter was sequentially rehybridized with 32P-labeled cDNA probes for rat pancreatic secretory PLA₂ (10, 14), murine IL-6 (14), and rat GAPDH (14). Autoradiographic densities were quantified by scanning densitometry (Scan Maker, Microtek, Torrence, CA) and appropriate software (Adobe Photoshop, Adobe Systems, Mountainview, CA; and Scan Analysis, Biosoft, Cambridge, UK). cPLA₂ mRNA abundance was corrected for background and normalized for changes in GAPDH transcript abundance.

**Protein phosphorylation and 35S labeling of cPLA₂ and immunoprecipitation.** Subconfluent mesangial cells in 100-mm plates were incubated for 1 h in serum-free, phosphate-free RPMI 1640 media, supplemented with 12.5 mM Hepes, pH 7.0, and NaHCO₃ (1.1 mg/ml). [32P]orthophosphate (0.35 mCi/10-cm dish) was added for an additional 2 h. The cells were stimulated with vehicle or IL-1 (50 ng/ml) for the final 30 min of labeling, washed twice with ice cold PBS and incubated for 10 min at 4°C in 0.4 ml of lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 30 mM sodium pyrophosphate, 0.2 mM sodium orthovanadate, and 1.0% NP-40). Exerts from three 10-cm dishes were combined and spun at 200,000 g for 60 min. The resulting supernatants from control and stimulated mesangial cell lysates were matched for protein, and cleared with protein A-Sepharose (Pharmacia). The samples (1 ml) were then incubated overnight at 4°C with excess (10 μl) rabbit antiserum against cPLA₂ or preimmune serum (7, kindly provided by Dr. James D. Clark). Immunocomplexes were precipitated using protein A-Sepharose, washed, and analyzed on 8% SDS-polyacrylamide gels. The 32P-labeled proteins were detected by autoradiography using intensifying screens. In some experiments, the extent of phosphorylation was quantitated using storage phosphor technology and quantification analysis (15). The storage phosphor imaging plate in a Molecular Dynamics (Sunnyvale, CA) exposure cassette for 2 d at room temperature and subsequently scanned by a Molecular Dynamics model 400A storage phosphor imager using a 10 MW helium-neon laser. Luminescence at 390 nm was collected, digitalized and stored. A digital image of the original incident radiation detected by the storage phosphor plates was generated, and bands of interest were quantified using Molecular Dynamics image quant software. Storage phosphor imaging plates have a large linear dynamic range and high sensitivity that allows accurate quantitative results (15). In some experiments, PLA₂ activity was quantified in cell lysates (110 μl) before and after cPLA₂ was immunoprecipitated using specific and preimmune antiserum (3 μl).

To assess cytokine-induced changes in de novo protein synthesis, cells were washed, depleted of methionine for 30 min and transferred to fresh methionine-, cysteine-free RPMI media containing [35S]-methionine (0.5 mCi/10-cm dish) for 1 h. IL-1 (50 ng/ml) was added for an additional 1 h. Cell-free lysates were prepared and immunoprecipitated, and immunoprecipitates were separated by electrophoresis as described above. 35S-labeled proteins were imaged by fluorography at ~70°C after soaking the gels with a fluoro amplifying reagent (Amer sham Corp.).

**Immunoblot analysis of cPLA₂.** 2 h before the experiment, cells maintained in complete medium were placed in RPMI media containing 10 mM Hepes, and treated with the indicated agonists. In some experiments, cells were serum-deprived for 24 h and then treated with IL-1 (10 ng/ml) or vehicle for an additional 24 h. Cells were then washed with PBS containing 4 mM EDTA, PMSF, leupeptin, and pepstatin A, and lysed as previously described (10). The samples (80 μg protein) were loaded on 8% SDS-polyacrylamide gel. To observe a phosphorylation-stimulated shift in cPLA₂ mobility (16), electrophoresis was continued until the 49-kD prestained protein marker (ovalbumin) had migrated to the end of the gel (a distance of 17 cm). The proteins were electrophoretically transferred (90 min at 85 V 4°C) to nitrocellulose membrane in a buffer containing 15% MeOH, 25 mM Tris, 192 mM glycine, 0.05% SDS, and 0.5 mM Na₂VO₄. The blot was blocked at 22°C for 2 h in PBS containing 0.2% Tween-20, 5% nonfat milk, and 0.5 mM Na₂VO₄. cPLA₂ protein was detected by incubation with a rabbit polyclonal antibody (1:2,000 dilution) against a recombinant fragment of human cPLA₂ (17) for 1 h followed by a 1 h incubation with a peroxidase-labeled, anti-rabbit IgG antibody (1:2,000 dilution, Kirkegaard & Perry, Gaithersburg, MD). The primary antibody cross-reacts with rat cPLA₂. Immunolocalized proteins were detected using a chemiluminescent detection system (ECL, Amer sham Corp.).
All blots were stained subsequently with India ink to verify that proteins were equivalent and transferred.

Results

IL-1 stimulates [³H]arachidonate release from intact cells. We first determined if IL-1 activated a PLA₂ before the previously described induction at 6–10 h of the group II sPLA₂. Enzyme activity was initially assessed by measuring intracellular and extracellular [³H]arachidonate release from equilibrum-labeled glomerular mesangial cells in the presence or absence of the calcium ionophore A23187. Calcium ionophore was included because IL-1 does not increase cytosolic Ca²⁺ (18) and most PLA₂s are Ca²⁺ dependent. IL-1 alone in incubations as long as 30 min did not significantly increase [³H]arachidonate release. In contrast, cells exposed to IL-1 followed by ionophore showed enhanced [³H]arachidonate release (Fig. 1). The small increase in arachidonate acid release detected at 2 min was followed by a statistically significant increase at 5 and 30 min. As a positive control, cells were also incubated with the phorbol ester PMA in the absence or presence of ionophore (Fig. 1). PMA is known to activate cPLA₂ in vitro and “primes” intact cells for enhanced arachidonate metabolism (4, 19). Primed stimulation is the synergistic enhancement of cellular responses, when combinations of stimuli are utilized. PMA alone had little effect on mesangial cellular [³H]arachidonate release but synergized with A23187 to stimulate a twofold increase in cytosolic arachidonate release (Fig. 1). These data suggest that IL-1, similar to PMA, primes cells for enhanced arachidonate release either by modifying a Ca²⁺-dependent PLA₂ or by activating a PLA₂ modulatory protein. Because IL-1 primes mesangial cells within 5 min for enhanced ionophore-stimulated arachidonate release, a PLA₂ that is distinct from the nonpancreatic group II sPLA₂ must be activated.

In vitro, the trifluoromethyl ketone analogue of arachidonic acid (AA-COCH₃) is a slow binding, but specific and potent inhibitor of cPLA₂ (11). We tested the effects of this compound in intact [³H]arachidonate-labeled cells. A 2-h preincubation with AA-COCH₃ completely inhibited IL-1-stimulated but not basal [³H]arachidonate release (Table I). Another arachidonate analogue, in which the trifluoromethyl ketone group is substituted with a -COCH₃ group (AA-COCH₃) and which is devoid of inhibitory activity, had no effect on IL-1-stimulated [³H]arachidonate release (Table I). Two inferences can be drawn from these data. First, IL-1 specifically activates cPLA₂ to stimulate arachidonic acid release in intact cells. Second, basal [³H]arachidonate release may result from a cPLA₂-independent mechanism since AA-COCH₃ had no effect in vehicle-stimulated cells (Table I), but almost completely inhibits cPLA₂ activity in the in vitro phosphatidyicholine vesicle assay using cell-free extracts (see below).

IL-1 stimulates cPLA₂ activity. Increased intracellular arachidonate may result from either activation of acylhydrolases and/or inhibition of acyltransferases (2). Since we and others have shown that IL-1 increases acyltransferase activity (20, 21), we focused on IL-1-stimulated decylation reactions in cell-free extracts. Mesangial cells were stimulated for 2 h with IL-1 (10 ng/ml) or vehicle in the absence of serum. As a positive control, extracts of cells stimulated with PMA (300 nM) for the final 10 min of serum deprivation, were assayed as shown in Table II. Both IL-1 and PMA stimulated PLA₂ activity in subconfluent mesangial cells. IL-1 consistently increased activity by twofold or greater. As previously reported (4), PMA gave a two- to threefold increase in PLA₂ activity. PLA₂ activity in extracts of cells incubated with IL-1 increased progressively from 10 to 60 min (Fig. 2A). At 30 min, IL-1 stimulated PLA₂ activity at concentrations between 1.0 and 10 ng/ml, and maximal activation was observed at 50–100 ng/ml (Fig. 2B). LPS contamination of reagents did not account for these results. cPLA₂ activity in extracts of control cells and cells treated with heated IL-1 was similar (not shown). Addition of polymyxin B, a binder of LPS, with IL-1 did not affect cPLA₂ activity assayed in the phosphatidyicholine vesicle assay.

These results clearly show that IL-1–treated mesangial cells rapidly activate an enzymatic activity which releases arachidonate from the sn-2 position of exogenous [¹⁴C]-phosphatidycholine in a standard assay for cPLA₂ activity. To characterize the IL-1–stimulated acylhydrolase activity, protein–matched cell extracts from control, IL-1–, and PMA–stimulated mesangial cells were fractionated on a Mono Q HR 5/5 column. The results of this separation indicate that the IL-1–stimulated activity is distinct from the cPLA₂ activity and is not due to any of the enzyme activities found in other peak fractions. These results are consistent with the idea that IL-1–stimulated PLA₂ activity is due to a PLA₂ distinct from the cPLA₂ enzyme.

Table I. [³H]Arachidonic Acid Release in the Presence and Absence of a Fatty Acid Analogue Inhibitory for cPLA₂

<table>
<thead>
<tr>
<th>Condition</th>
<th>[³H]Arachidonic acid released</th>
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<tr>
<td></td>
<td>cpm</td>
</tr>
<tr>
<td>Inactive FA analogue (AA-COCH₃)</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>10,781±486</td>
</tr>
<tr>
<td>IL-1</td>
<td>17,635±548</td>
</tr>
<tr>
<td>Inhibitory fatty acid analogue (AA-COCH₃)</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>9,889±913</td>
</tr>
<tr>
<td>IL-1</td>
<td>10,809±1078</td>
</tr>
</tbody>
</table>

Mesangial cells were labeled with [³H]arachidonate as described in Fig. 1. The cells were deprived serum for 2 h in the presence of the inhibitory fatty acid or the inactive fatty acid analogue. (Final concentrations, 25 μM.) Cells were then stimulated with vehicle or IL-1 (20 ng/ml) for 20 min. A23187 (0.25 μM) was subsequently added for 10 min. [³H]arachidonate release was quantified as described in Fig. 1. Results are the mean±SD of triplicate wells representative of duplicate experiments. Total [³H] incorporation was the same in control and IL-1–stimulated cells in both treatment protocols.

Table II. Stimulation of cPLA₂ Activity in Rat Mesangial Cells by IL-1 and PMA

<table>
<thead>
<tr>
<th>Condition</th>
<th>PLA₂ activity (pmol/min/mg)</th>
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<tbody>
<tr>
<td>Control</td>
<td>238.9±27.9</td>
</tr>
<tr>
<td>IL-1</td>
<td>497.8±99.4*</td>
</tr>
<tr>
<td>PMA</td>
<td>664.3±92.8†</td>
</tr>
</tbody>
</table>

Subconfluent cultures of mesangial cells were incubated in serum-free media for 2 h, with or without IL-1 (10 ng/ml). In some cultures, PMA (300 nM) was added for the final 10 min of the serum-free incubation. Cell-free extracts were prepared by homogenization and ultracentrifugation as described in Methods, matched for protein, and assayed for PLA₂ activity. Results are expressed as mean values±SEM for triplicate determinations in four individual experiments. * P < 0.025, † P < 0.005 compared to the control value.

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anion exchange column. PLA₂ activity was eluted with a NaCl gradient. > 95% of enzyme activity bound to the column. As shown in Fig. 3, the PLA₂ activity from each extract comigrated as a single peak, which was eluted with 0.4-0.45 M NaCl. This elution profile is consistent with that previously described for cPLA₂ in U937 and mesangial cells (4-6, 12). PLA₂ activity in fractionated extracts from both IL-1- and PMA-treated cells maintained higher specific activities than control extracts. IL-1-stimulated sPLA₂ is eluted with 1.2 M NaCl (M. Konieczkowski and J. R. Sedor, unpublished results). No PLA₂ activity was detected in fractions eluted with this salt concentration. Recoveries in all cases were > 75%.

As a complementary approach to verify that the IL-1-stimulated PLA₂ activity was cPLA₂, we incubated IL-1-stimulated extracts for 30 min at room temperature with either a rabbit polyclonal antiserum against human cPLA₂ (7) or preimmune antiserum. The specific antiserum recognizes rat cPLA₂ epitopes. After immunoprecipitation, residual supernatant PLA₂ activity was undetectable in control or IL-1-stimulated mesangial cell extracts. In contrast, PLA₂ activity was similar before and after immunoprecipitation with preimmune serum in extracts of IL-1-stimulated cells (control extracts, 141.1±12.7 pmol/min per mg; untreated IL-1-stimulated extracts, 238.5±13.6 pmol/min per mg; and residual cPLA₂ activity in IL-1-stimulated extracts pretreated with preimmune sera, 310.2±21.5 pmol/min per mg, n = 2).

The effects of several PLA₂ inhibitors on PLA₂ activity were tested in the phosphatidylethanolamine vesicle assay. The specific cPLA₂ inhibitor (11), AA-COCF₃, completely inhibited IL-1-stimulated cPLA₂ activity. In contrast, a control arachidonate analogue, AA-COCH₃, which is devoid of inhibitory activity (11), had no effect. cPLA₂ activity was 72.1±5.77, 720.8±33.8, and 95.0±9.2 pmol/mg per min (mean±SEM, n = 3) in vehicle (DMSO)-, AA-COCH₃ (10 μM)-, and AA-COCF₃ (10 μM)-treated extracts of IL-1-stimulated mesangial cells. Compounds that effectively inhibit sPLA₂ activation, less effectively inhibited cPLA₂ activity in extracts of both control and IL-1-stimulated cells. Aristolochic acid (150 μM), quinacrine (100 μM), and 7, 7-dimethyl-5,8-epicosadienoic acid (5 μM) inhibited IL-1-stimulated activity by 36±4.1%, 28±2.8%, and 18±0.3%, respectively. A qualitatively similar pattern of inhibition was observed when these compounds were added to vehicle-stimulated cells (not shown). These data provide further evidence that IL-1 specifically can activate cPLA₂.

Other biochemical characteristics of the cytokine-activated cPLA₂ were determined. Unlike the IL-1-induced sPLA₂ activity (10), the PLA₂ activity rapidly stimulated by IL-1 hydrolyzed arachidonate from both [³¹C]phosphatidylcholine and [¹⁴C]phosphatidylethanolamine (data not shown). A pH (4.5-9.0) dependence analysis of the IL-1-stimulated cPLA₂ demonstrated little activity below pH 7.0 and peak activity at an alkaline pH (approximately pH 9.0). Significant PLA₂ activity could not be measured in the extracts in the absence of Ca²⁺ of either vehicle- or IL-1-stimulated mesangial cells (data not shown). These characteristics are similar to those previously reported for both the mesangial cell and kidney enzymes (4, 12, 22).

Regulation of cPLA₂ by IL-1. The preceding data suggest that IL-1 causes a stable modification of cPLA₂ since the cytokine-activated enzyme stimulation was maintained after cell disruption, ultracentrifugation, and column fractionation. We explored two potential mechanisms which could result in a stable enhancement of cPLA₂ activity: (a) de novo synthesis of cPLA₂ or a stimulatory protein such as PLA₂-activating protein, and (b) a posttranslational modification of cPLA₂ which increases enzyme activity.

As shown in Fig. 4, Northern analysis demonstrated cPLA₂ mRNA in both control and cytokine-treated mesangial cells maintained in 17% serum. In four separate experiments, IL-1 had no consistent effect on cPLA₂ mRNA expression or activity (not shown) in incubations as long as 24 h, but induced expression of sPLA₂ at 24 h (Fig. 4) and IL-6 at 1 and 2 h (not
Moreover, IL-1 did not induce cPLA₂ protein when newly synthesized proteins in vehicle- and IL-1-stimulated (1 h) mesangial cells were radiolabeled with [35S]methionine and enzyme levels were analyzed by immunoprecipitation (data not shown). Taken together, these data demonstrate that the rapid stimulation of cPLA₂ activity in serum-stimulated cells by IL-1 did not result from increased enzyme mass.

To assess whether the cytokine-stimulated cPLA₂ activity resulted from de novo synthesis of a PLA₂-activating protein, cycloheximide was added for 30 min or 2 h before the addition of IL-1 (50 ng/ml) for a subsequent 30 min incubation. Cycloheximide (2 μg/ml) pretreatment had no effect on IL-1-stimulated cPLA₂ activity. The percent stimulation of enzyme activity by IL-1 above the control value was 239±78 and 211±37 (mean±SEM, n = 2) in the absence and presence of cycloheximide, respectively.

We next determined whether IL-1-stimulated cPLA₂ activity resulted from a post-translational modification, and hy-
pothesized that IL-1 altered enzyme activity by phosphorylation for two reasons. First, cPLA2 contains consensus phosphorylation sequences for several kinases (7, 16); second, IL-1 activates serine-threonine-kinases (23, 24). Mesangial cells were labeled in the absence of serum with [32P]orthophosphate for 2 h and stimulated with IL-1 (50 ng/ml) for an additional 30 min. cPLA2 was immunoprecipitated from the high-speed supernatants of control and IL-1-treated cells using polyclonal cPLA2 antiserum or preimmune sera. As shown in Fig. 5A, a specific protein was precipitated by the anti-cPLA2 antiserum but not preimmune serum in both the control and IL-1-treated cells. The specific band was 97 Kd, corresponding to the molecular mass of phosphorylated cPLA2. 32P incorporation into this band was nearly twofold greater than control in response to IL-1, a finding demonstrated in duplicate experiments. This IL-1-induced increase in cPLA2 phosphorylation correlated with the magnitude of enzyme activation by IL-1 in whole cells at 30 min and is consistent with the idea that protein phosphorylation may regulate cPLA2 activity. Others have demonstrated that agonist-induced phosphorylation and activation of cPLA2 is associated with decreased mobility in SDS-polyacrylamide gels (16). Immunoblot analysis demonstrated that treatment of mesangial cells with IL-1 for 30 min caused shift in cPLA2 mobility from a more rapidly to a more slowly migrating species (Fig. 5B). As previously described (16), PMA similarly induced a shift in cPLA2 mobility.

We next determined if phosphorylation of cPLA2 by IL-1 resulted in increased enzyme activity (Table III). cPLA2 activity was twofold higher in high-speed supernatants derived from IL-1-stimulated cells compared to vehicle-treated cells. In two separate experiments, treatment of the extracts with human acid phosphatase (0.4 IU) significantly reduced IL-1-stimulated enzyme activity but had no significant effect on enzyme activity in control cells. Taken together, these data suggest that IL-1 enhances cPLA2 by phosphorylation. Basal cPLA2 activity either is not dependent on phosphorylation or results from a phosphorylation event which is insensitive to acid phosphatase.

Mesangial cells used in the preceding experiments were either serum stimulated or acutely serum deprived (2 h). For two reasons, we next assessed IL-1-induced cPLA2 expression in cells that were held for 24–48 h in 0.5% FBS. First, we have demonstrated that growth factors in serum attenuate induction of sPLA2 mRNA activity (14). Second, serum contains glucocorticoids and dexamethasone prevents IL-1 induction of cPLA2 in other cell types (25, 26). Fig. 6 depicts the results of a representative Northern and immunoblot analysis of cPLA2 expression in control and IL-1-stimulated, serum-deprived cells. IL-1 increased cPLA2 mRNA in 24 h incubations (Fig. 6 A) but not in incubations shorter than 6 h (not shown). Relative cPLA2 mRNA levels, corrected for changes in GAPDH mRNA, were 2.4±0.4-fold (mean±SEM, n = 2) higher in IL-1-stimulated cells when compared to mesangial cells only exposed to vehicle. IL-1 concordantly increased cPLA2 protein 1.7±0.1-fold (mean±SEM, n = 2) (Fig. 6 B). Under these conditions, cPLA2 mobility is similar in vehicle- and IL-1-stimulated cells, suggesting that the amounts of hormones/growth factors contained in 0.5% of serum can maintain cPLA2 in its phosphorylated state. Consistent with this hypothesis, we have noted that mesangial cells must be completely serum-deprived to maximally down-regulate cPLA2 activity (M. Konieczkowski and J. R. Sedor, unpublished results). cPLA2 enzyme activity was similarly doubled in serum-deprived mesangial cells treated with IL-1 for 24 h (385.6±13.2 and 620.3±15.2 pmol/min per mg; extracts from control and IL-1-treated cells, mean±SEM, triplicate determinations). This finding is consistent with our previous results using the phosphatidylinositol vesicle assay (10). Although IL-1 markedly induces sPLA2 by 24 h, phosphatidylinositol vesicles are a poor substrate for
Table III. cPLA2 Activity in the Presence and Absence of Acid Phosphatase Treatment

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Stimulus</th>
<th>cPLA2 activity (pmol/min per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Control (6)</td>
<td>322.1±33.3</td>
</tr>
<tr>
<td></td>
<td>IL-1 (6)</td>
<td>633.1±79.2</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>Control (9)</td>
<td>245.3±22.6*</td>
</tr>
<tr>
<td></td>
<td>IL-1 (7)</td>
<td>331.1±68.3*</td>
</tr>
</tbody>
</table>

0.4 U of human acid phosphatase (Calbiochem Corp., 500 IU/ng) was added to 2.5–5 µg of mesangial cell extract prepared as described in Methods. The extracts and acid phosphatase were incubated for 15 min at 30°C, followed by the addition of 30 nCi [3H]arachidonyl-phosphatidylethanolamine and 5 mM CaCl2. The samples were incubated subsequently for an additional 30 min at 37°C, and the cPLA2 activity was quantified as described. cPLA2 activity is expressed as the mean±SEM for triplicate determinations in two separate experiments. IL-1 significantly stimulated cPLA2 activity over control. * P < 0.01, IL-1 stimulated vs. IL-1 stimulated and acid phosphatase pretreated; † P > 0.1, vehicle stimulated vs. vehicle stimulated and acid phosphatase pretreated.

this PLA2 isomor, suggesting the changes in PLA2 activity measured by this assay if not exclusively a result of cPLA2 activation.

Discussion

IL-1 is a potent stimulus of eicosanoid metabolism (1, 9, 10). In the present study, we have demonstrated that IL-1, in the presence of ionophore, quickly stimulates arachidone release from intact mesangial cells. In whole-cell lysates, IL-1 rapidly activates a PLA2 isomor which we have characterized as cPLA2. This increase in cytokine-stimulated cPLA2 activity does not require new protein synthesis but is calcium dependent. IL-1 doubles 32P incorporation into immunoprecipitable cPLA2 protein consistent with the fold stimulation of enzyme activity in similarly timed incubations. Importantly, the IL-1-stimulated enhancement of PLA2 activity was significantly reduced by acid phosphatase treatment. Taken together, these data suggest that IL-1-stimulated eicosanoid metabolism is mediated, at least acutely, by phosphorylation and activation of cPLA2. This supports the premise, advanced by Lin et al. (16), that agonist-stimulated increases in intrinsic cPLA2 activity are only apparent when a rise in cytosolic Ca2+ allows translocation of the enzyme to the cytosol (4, 7, 27). IL-1 does not increase cytosolic Ca2+ in mesangial cells (18) and by itself does not increase arachidonate release (10, this paper). In contrast, arachidonic acid release stimulated by the Ca2+ mobilizing agonists, vasopressin (10) and ionophore (this paper), is doubled in IL-1-stimulated cells.

Previous studies have suggested that PLA2 activity can be regulated by changes in its phosphorylation state. Sequence analysis of cPLA2 has demonstrated consensus phosphorylation sites for the mitogen-activated protein (MAP) kinase and protein kinase C (7, 16), and cPLA2 activity can be both positively and negatively regulated by agents that increase activity of certain kinases. Protein kinase C activators prime or potentiate agonist-induced arachidonate release, an effect that can be blocked in part by either protein kinase C down-regulation or PKC inhibitors (19, 28–30). EGF, similar to IL-1 and PMA, causes an increase in cPLA2 activity that results from stable posttranslational modification (4, 12), and EGF receptor tyrosine kinase activity and autophosphorylation appears necessary for PLA2 activation (31–33). One study has suggested that PLA2 is directly activated by the EGF receptor tyrosine kinase (34). Calcium-dependent PLA2 activity can be potentiated by the addition of either the catalytic subunit of the cAMP-dependent protein kinase or by casein kinase II and is inhibited by incubation with the Ca2+/calmodulin-dependent protein kinase II (35). Lin and co-workers have reported that treatment of CHO cells, overexpressing cPLA2 with ATP, A23187, PMA, and thrombin increases arachidonate release from whole cells, stimulates cPLA2 activity in vitro, and concomitantly increases 32P incorporation into cPLA2 exclusively on serine residues (16). The protein kinase C inhibitor staurosporine prevents ATP-stimulated cPLA2 phosphorylation and activation, and treatment of ATP-stimulated cell lysates with acid phosphatase eliminates agonist-induced increases in cPLA2 activity. Recent data in vitro has suggested that both MAP kinase and protein

Figure 6. Northern (A) and immunoblot (B) analysis of cPLA2 mRNA and protein, respectively, in vehicle and IL-1-stimulated mesangial cells. Cells were held in medium containing 0.5% FBS for 24 h and subsequently stimulated with IL-1 for 24 h. cPLA2 mRNA and protein expression were determined as described in Methods.
kinase C can activate cPLA₂ by phosphorylation (36, 37). Collectively, these data strongly implicate posttranslational changes in cPLA₂ phosphorylation state as an important regulator of enzyme activity.

Since cell lysates rather than purified protein have been used as the enzyme source, our data, as well as most of the data just summarized, are also consistent with activation of a cPLA₂ regulatory protein by phosphorylation. PLA₂ stimulatory proteins have been isolated from mammalian cells. Best characterized is a 28-kd protein, PLA₂-activating protein (PLAP) (38). Rapid, agonist-stimulated, de novo synthesis of PLAP is the primary mechanism of PLA₂ activation by PLAP (38). However, we found that cycloheximide does not prevent IL-1-stimulated cPLA₂ activation. A PLAP has also been purified from Aplysia (39). This PLA₂ stimulatory activity is increased in Aplysia extracts treated with phorbol dibutyrate, and in vitro, this protein is a substrate for protein kinase C (39). Phosphorylation also inactivates or enhances the degradation of lipocortins, molecules which bind to phospholipids and prevent PLA₂ activation by "substrate depletion." However phosphorylation of lipocortin I does not correlate temporally with EGF-stimulated activation of PLA₂ in cells overexpressing the wild-type EGF receptor (33). In vitro reconstitution experiments have demonstrated that both MAP kinase and protein kinase C phosphorylate and increase cPLA₂-specific catalytic activity (36, 37), suggesting that direct phosphorylation of cPLA₂ stimulates, at least in part, increased enzymatic activity in intact cells.

IL-1-dependent protein phosphorylations have been reported previously, and IL-1 stimulates protein serine-, threonine-kinase activity in a variety of cell types. The identity of the kinase(s) involved in IL-1-mediated transmembrane signalling is still speculative. IL-1-stimulated arachidonate release in fibroblasts does not require either protein kinase C or cAMP-dependent protein kinase activity (40). Cleveland maps of phosphorylated talin in fibroblasts demonstrate distinct phosphopeptides after PMA and IL-1 treatment, suggesting that the IL-1 activates kinases distinct from C kinase (41). Recently, IL-1 has been shown to rapidly stimulate MAP kinase activity (23, 24) and one study has suggested that IL-1 increases protein phosphorylation by inhibition of protein phosphatase activity (42). cPLA₂ is phosphorylated and activated in vitro by MAP kinase (36, 37), and we suggest that IL-1-stimulated MAP kinase phosphorylates cPLA₂ to increase its activity. Phosphorylation of distinct cPLA₂ domains may independently regulate enzyme activity. IL-1 and vasopressin synergistically increase arachidonate release; vasopressin increases protein kinase C activity in mesangial cells but IL-1 does not. In vitro protein kinase C and MAP kinase phosphorylate distinct residues within recombinantly expressed cPLA₂ domains (37). However other workers have suggested protein kinase C and MAP kinase identically phosphorylate the same amino acid residue (36).

Stimulation of PLA₂ activity by IL-1 may be cell specific. Similar to our findings, IL-1 increased a PLA₂ activity, with Ca²⁺ requirements similar to cPLA₂, in rheumatoid synovial fibroblasts (43). Treatment of a human lung fibroblast line with IL-1 results in cPLA₂ phosphorylation (25). However, in contrast to our findings in mesangial cells (10, this paper), IL-1 only stimulated PGE₂ synthesis in fibroblasts after prolonged (> 5 h) incubation and did not induce sPLA₂ activity (25). Human IL-1β added to guinea pig cosinophils negatively regulated PLA₂ activity through an indirect, Ca²⁺ independent mechanism (44). In a preliminary report, Clark and coworkers have demonstrated that de novo PLAP synthesis mediates IL-1-stimulated PLA₂ activity in EL-4 cells (45). This cell specific regulation of IL-1-stimulated PLA₂ activity suggests that arachidonic acid hydrolysis may be an important determinant in the cellular response to inflammation.

cPLA₂ activity clearly is regulated at multiple levels. In addition to a rapid posttranslational modification of cPLA₂, IL-1 induces, in 24-h incubations a small (approximately twofold) but consistent increase in cPLA₂ mRNA, protein, and activity. This effect was only detected in mesangial cells held in 0.5% FBS but not those cells maintained in serum. Growth factors (14) and/or glucocorticoids (25, 26) present in serum may negatively regulate IL-1-induced cPLA₂ expression. Long-term incubations (> 8 h) with macrophage colony-stimulating factor (46). TNF (25), epidermal growth factor (47) and PMA (47) also increases cPLA₂ expression. cPLA₂ induction by IL-1 and TNF is not surprising, in that both cytokines activate the transcriptional factors, AP-1 and NFκB, and the 5′ flanking region of the cPLA₂ gene contains AP-1 and κB binding elements (48).

In summary, IL-1 regulates the activity of cPLA₂ in a bi- phasic, time-dependent manner. A rapid increase in catalytic activity results from protein phosphorylation. More prolonged incubation with IL-1 can increase cPLA₂ mass in resting but not serum-activated cells. cPLA₂ activation provides one mechanism for biological events induced by this cytokine. Arachidonic acid release and enhanced eicosanoid metabolism are hallmarks of glomerular inflammation. Increased prostaglandin, HETE, and leukotriene synthesis mediate both the hemodynamic alterations and the cellular activation which characterize inflammatory injury. Recent studies also have demonstrated that these same fatty acid mediators comprise an important intracellular, transmembrane signaling system. Arachidonic acid and/or its metabolites activate GTP-binding proteins (49), regulate the activity of Ras-GTPase activating protein (50), stimulate kinase activity (51), control ion channel function (52), and can activate latent transactivating factors (53). These interactions between arachidonate and other second messenger pathways provide mechanisms by which IL-1-mediated activation of cPLA₂, not only results in the synthesis of proinflammatory lipid mediators, but also initiates a specific program of phenotypic responses in the mesangial cell.

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