# Cell-specific Regulation of Type II Phospholipase A2 Expression in Rat Mesangial Cells

**M. Konieczkowski and J. R. Sedor***

*Departments of Medicine and Physiology and *Biophysics, School of Medicine, Case Western Reserve University and Divisions of Nephrology, MetroHealth Medical Center and University Hospitals, Cleveland, Ohio 44109*

## Abstract

**IL-1 stimulates mesangial cells to synthesize specific proteins, including a non-pancreatic (Type II) phospholipase $A_2$ ($PLA_2$). We have studied the regulation of $PLA_2$ by proinflammatory mediators, implicated in the pathogenesis of glomerulonephritis, and have assessed whether the activation of second messenger systems modulates or mimics $PLA_2$ gene expression by cytokines. IL-1$\alpha$ and $\beta$, TNF$\alpha$, and LPS, but not serum, IL-2, or PDGF, potently induce $PLA_2$ mRNA, and enzyme expression. IL-1-stimulated mesangial cells express a 1.0 kB $PLA_2$ mRNA transcript that is induced in a dose- and time-dependent manner. IL-1-stimulated increases in steady-state $PLA_2$ mRNA abundance result from a moderate increase in $PLA_2$ transcription rate that is amplified by the prolonged persistence of the transcript. Forskolin and dibutyryl cAMP potentiate IL-1-induced $PLA_2$ mRNA and enzyme expression, but have no effect in the absence of cytokine. 12-tetradecanoyl phorbol 13-acetate, *sn*-1, 2-dioctanoyl glycerol or 1-oleoyl-2-acetyl-*sn*-glycerol fail to induce $PLA_2$ expression or to alter the effect of IL-1 when coincubated with the cytokine. In contrast, serum deprivation for 24 h specifically enhances IL-1-stimulated $PLA_2$. Genistein potentiates $PLA_2$ mRNA expression in cells exposed to both IL-1 and serum. The inhibitory effect of serum on IL-1-induced $PLA_2$ mRNA abundance is reproduced by PDGF but not dexamethasone. These data demonstrate that the signaling pathways directly engaged by IL-1 to induce $PLA_2$ expression in mesangial cells interact with several second messenger systems in a cell-specific manner. We speculate that IL-1 induces specialized changes in mesangial cell structure and function through direct activation of a transcription factor(s), that result in induction of a specific gene set. (*J. Clin. Invest.* 1993. 92:2524–2532.) Key words: cell phenotype • cell differentiation • interleukin-1 • glomerulonephritis • cytokine**

## Introduction

The mesangial cell occupies a central anatomical position within the glomerulus (1, 2). Historically this cell has been regarded as unifunctional, only being important in the regulation of glomerular filtration rate. However, in vitro and in vivo evidence suggests that the mesangial cell both produces and responds to biological modifiers and participates in the destructive and restorative processes that follow the initiation of glomerular injury (1, 2). Intraglomerular expression of the cytokines, IL-1 and TNF $\alpha$[1] has been demonstrated in both human and experimental glomerulonephritis (3, 4). IL-1 and TNF activate the mesangial cell in culture to express new structural and functional characteristics in part by inducing expression of specific genes (3, 4). IL-1$\alpha$, IL-1$\beta$, and TNF stimulate release of the neutrophil chemotactic intercrine, IL-8 (5–7), and IL-6 (6, 7) and regulate expression of genes controlling extracellular matrix synthesis and turnover (3, 4, 8). We have demonstrated in rat mesangial cells that IL-1$\alpha$ directly stimulates prostaglandin synthesis by increasing phospholipase $A_2$ ($PLA_2$)[1] activity through synthesis of a nonpancreatic (Type II) $PLA_2$ (9). The increases in $PLA_2$ activity and $PLA_2$ mRNA expression are coordinate and follow the same time course as cytokine-stimulated $PGE_2$ synthesis.

As a result of these and other studies, many investigators have hypothesized that TNF- and IL-1-stimulated mesangial cells express an altered phenotype that contributes to glomerular injury. The intracellular events that mediate the cytokine-induced transition of mesangial function are poorly understood. This process involves induction of a specific gene set by a network of short-term signals generated by interaction of the cytokine with the binding domain of its receptor. The cytosolic signals generated by IL-1 and TNF in the mesangial cell have been fairly well defined. We and others have shown that IL-1$\alpha$ rapidly stimulates 1, 2-diacylglycerol and phosphatidic acid formation (10, 11) but does not induce rapid changes in cystolic $Ca^{2+}$ or $pH_i$ (10). Both IL-1 and TNF cause phosphorylation of mesangial cell proteins, but neither cytokine increases the phosphorylation of the putative 80 kD substrate of protein kinase C (3, 12). Pfeilschifter shows no increase in cAMP accumulation in response to IL-1 (13), and TNF and IL-1 only stimulate cGMP formation after 4 to 6 h (14, 15).

We have used the induction of $PLA_2$ mRNA expression by IL-1 as a model system to define whether these early signals are converted to long-term changes in cell phenotype through induction of specific genes. Our studies suggest that the signalling pathways directly engaged by IL-1 in the mesangial cells interact with several second messenger systems in a cell-specific manner.

## Methods

*Materials.* Recombinant human IL-1$\alpha$ (rIL-1), and TNF were kindly provided by Drs. Peter Lomedico (Hoffmann-LaRoche, Nutley, NJ)

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Address correspondence to Dr. John R. Sedor, Division of Nephrology, MetroHealth Medical Center, 2500 MetroHealth Drive, Office R-338, Cleveland, OH 44109-1998.

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1. *Abbreviations used in this paper:* DiOG, *sn*-1, 2-dioctanoylglycerol; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PA, phosphatidic acid; $PLA_2$, phospholipase $A_2$; rIL-1, recombinant human IL-1$\alpha$; TPA, tetradecanoyl phorbol 13-acetate.

and Leo Linn (Cetus Corporation, Emeryville, CA). Specific activities and endotoxin contents have previously been reported (9, 10, 16). Recombinant human interleukin-1$\beta$ ($2 \times 10^7$ U/mg) was a gift from Dr. Nigel Staite (UpJohn, Kalamazoo, MI), and endotoxin concentration determined by limulus lysate assay was 1.8 ng/mg protein. Stock cytokines were diluted 1:50,000 before use with pyrogen-free saline containing 2 mg/ml lipopolysaccharide-free BSA (Calbiochem-Novabiochem Corp., San Diego, CA). Recombinant human platelet derived growth factor (PDGF, BB homodimer) was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. Tetradecanoyl phorbol 13-acetate (TPA), forskolin, genistein, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) and *sn*-1, 2-dioctanoylglycerol (diOG) were from Calbiochem-Novabiochem Corp. LPS, dibutyryl cAMP and arachidonic acid were supplied by Sigma Chemical Co. (St. Louis, MO). L-$\alpha$-1-palmitoyl, 2-arachidonyl[arachidonyl-1-$^{14}$C]-phosphatidylethanolamine (60 mCi/mmol), and uridine 5′-triphosphate, [$\alpha$-$^{32}$P] (800 Ci/mmol) were purchased from DuPont-New England Nuclear (Boston, MA). Phosphatidic acid, derived from lecithin, was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Silica gel 60 thin layer chromatography plates (250 $\mu$m thickness) were from E. M. Science (Gibbstown, NJ).

*Cell culture.* Well characterized mesangial cells were grown from collagenase-treated rat glomeruli as previously described (9, 10, 16, 17). Experiments were performed on near confluent cells in passages three to eight. For most studies, cells were incubated in 12.5 mM Hepes buffered RPMI 1640 media containing 5% FBS (Hyclone Laboratories, Logan, UT). In some experiments, cells were incubated in media lacking FBS (designated 0% FBS) which contained 0.05% BSA (Sigma Chemical Co.) or 0.1% pyrogen-free human serum albumin (Armour Pharmaceutical, Kakakee, IL). In other experiments cells were serum-restricted for 3 d in RPMI 1640 (M.A. Bioproducts, Walkerville, MD) containing 0.5% FBS, washed and exposed to PDGF or vehicle.

*$PLA_2$ activity and mRNA levels.* $PLA_2$ activity was assayed in acid extracts of vehicle- and cytokine-treated mesangial cells exactly as we have published (9). Briefly, mesangial cells cultured in 75 cm$^2$ flasks were scrapped and homogenized in 0.18 N $H_2SO_4$ and 1 M NaCl, and the cleared supernatants were dialyzed overnight against 10 mM sodium acetate, pH 4.4. In some experiments, medium (0% FBS) was removed and centrifuged briefly at 12,000 *g* to remove cellular debris. The extracts and media were stored at −70°C until assay. $PLA_2$ activity was assayed in vehicle and rIL-1-treated mesangial cell homogenates (15–30 $\mu$g protein) and media (50 $\mu$l) using exogenous [$^{14}$C]-arachidonyl-phosphatidylethanolamine (final concentration, 16 $\mu$M) as substrate in the presence of 100 mM Tris HCl, pH 8.5, and 2.5 mM $CaCl_2$ for 60 min at 37°C. Hydrolyzed arachidonic acid was separated from the phospholipid substrate using silica gel thin layer chromatography. The characteristics of $PLA_2$ activity released into the medium are identical to those we have reported for acid-extracted activity (9). Released $PLA_2$ activity is calcium-dependent (99% reduction by inclusion of 10 mM EGTA in the assay buffer), is inhibited by greater than 85% by 100 mM *p*-bromophenacyl bromide and represents more than 90% of the combined acid extractable and secreted $PLA_2$ activity. Results presented in the figures are shown as the mean±SEM value of duplicate determinations from the indicated number of experiments.

Northern analysis of total cellular RNA (25 $\mu$g) from vehicle- and rIL-1 stimulated cells was used to size the $PLA_2$ as previously described (5, 9, 17). Total mesangial cell RNA was extracted using 4 M guanidine isothiocyanate and purified by ultracentrifugation through a cesium chloride cushion. Rat spleen RNA was concurrently analyzed as a positive control. The filters were hybridized at 42°C overnight with a full length $PLA_2$ cDNA probe (2 ng/ml, provided by Dr. Hiroshi Teraoka, Shionogi and Co, Ltd, Osaka, Japan) labeled by nick translation. The membranes were subsequently washed for 30 min at room temperature with 2 × SSPE (1 × SSPE:180 mM NaCl, 10 mM $Na_2HPO_4 \cdot 7H_2O$, and 1 mM EDTA), for 30 min with 2 × SSPE, 2% SDS at 50°C, and for 15 min with 0.1 × SSPE, 1% SDS at 52°C. Autoradiograms were obtained by exposing the blots to Kodak XAR-5 film overnight. Transcript size (kb) was derived from an mRNA ladder of known fragment sizes (Bethesda Research Laboratories, Gaithersburg, MD). The filters were stripped and rehybridized with a $^{32}$P-labeled chicken $\alpha$-tubulin cDNA probe. IL-6 mRNA expression was also assayed using the hybridization conditions described above. A $^{32}$P-labeled 650 bp cDNA fragment from murine IL-6 (gift from Dr. Steven Clark, Genetics Institute, Cambridge, MA) was used as a probe in some studies.

$PLA_2$ mRNA abundance was assessed by RNA-RNA solution hybridization as we have previously described in detail (9). Briefly, T7 RNA polymerase was used to synthesize a [$^{32}$P]cRNA probe using a Pvu II-linearized DNA template that contained a cDNA sequence (bp 381–758) for rat splenic, nonpancreatic (Type II) $PLA_2$. A heterogenous nucleotide residue found in position 461 of the mature $PLA_2$ RNA transcript produces protected transcripts of both 377 nucleotide and 297 nucleotide (9). For this study, we have constructed, by standard methodologies, an additional DNA template that contains bp 666–759 of the $PLA_2$ cDNA. T7 RNA polymerase was used to synthesize a 340 nt $^{32}$P-labeled probe from this template, which, when hybridized to total cellular RNA, protected a 93 nt transcript. Total mesangial cell RNA and the indicated probes were hybridized at 49°C overnight. Following hybridization, unprotected single stranded RNA was digested using RNAse A (50 $\mu$g/ml) and T1 (2 $\mu$g/ml) for 30 min at 30°C for the first [$^{32}$P]$PLA_2$ probe described and for 25 min at 22°C for the second. [$^{32}$P]RNA-RNA hybrids were analyzed on 5% or 8% denaturing polyacrylamide gels and detected by autoradiography (exposures 1–3 d). Transcript abundance was quantitated by densitometry and corrected for background (yeast RNA). Differences between mean $PLA_2$ mRNA expression were analyzed for statistical significance using Student's *t* test. In some experiments, a $^{32}$P-labeled RNA probe for human $\alpha$-tubulin (provided by Dr. D. Goldthwait, Department of Biochemistry, Case Western Reserve University, Cleveland, OH) was included in the hybridization reactions to assess sample loading and specificity of changes in $PLA_2$ mRNA expression. A 389 nt $^{32}$P-labeled transcript was synthesized from a cDNA template that contained a 135 nt sequence of a human $\alpha$-tubulin (1–135 bp). Rat and human $\alpha$-tubulin mRNA are homologous only in their coding region, resulting in a 67 nt protected fragment. Autoradiograms representative of two to four separate experiments are shown.

*Nuclear run-off transcription assay.* The procedure for in vitro labeling of nuclear RNA was taken from published methods (18) and modified as indicated. Confluent mesangial cells ($1.5 \times 10^7$ cells/condition) were incubated in 0% FBS without or with rIL-1 (10 ng/ml) for 15 h. Nuclei were isolated by lysis in 0.5% NP-40 using two cycles of dounce homogenization and resuspended in 200 $\mu$l of nuclear freezing buffer (40% glycerol, 50 mM Tris HCl, pH 8.3, 5 mM $MgCl_2$, and 0.1 mM EDTA). The transcription assays were initiated by adding 100 $\mu$l of 3X transcription buffer (15 mM Tris HCl, pH 8.0, 15 mM $MgCl_2$, 360 mM KCl, 7.5 mM DTT, 1.5 mM each of ATP, CTP, GTP, and 0.2 $\mu$M UTP along with 300 $\mu$Ci of [$^{32}$P]UTP [3,000 Ci/mmol; Dupont-NEN]) to the freshly isolated nuclei. In some cases, the nuclear preparations were preincubated with $\alpha$-amanitin (1 $\mu$g/ml) for 10 min before the start of the transcription assay. After 60 min at 28°C, the reaction was terminated by the addition of 100 U of DNase I in 300 mM NaCl, 30 mM $MgCl_2$, 1.2 mM $CaCl_2$, and 6.0 mM Tris, pH 7.4. This suspension was treated subsequently for 30 min at 37°C with proteinase K (200 $\mu$g) in 1% SDS, 5 mM EDTA, and 10 mM Tris, pH 7.4, (1 × SET), extracted with phenol/chloroform/isoamyl alcohol and precipitated overnight at 20°C with isopropanol. The precipitate was resuspended in 50 $\mu$l of 1 × SET and centrifuged through a G-25 Sephadex spin column (Quick Spin; Boehringer Mannheim Biochemicals). The purified RNA was subjected to limited base hydrolysis in 0.2M NaOH for 7 min at 4°C, neutralized with 0.48 M Hepes (free acid), and reprecipitated. Greater than 90% of the [$^{32}$P]cpm were precipitated with 10% trichloroacetic acid. The resulting pellet was resuspended in 10 mM N-tris [hydroxymethyl]methyl-2-aminoethane sulfonic acid (TES), pH 7.4, 10 mm EDTA, 0.2% SDS, and 0.3 M NaCl (TES/NaCl). Equivalent radioactive RNA ($5 \times 10^6$ cpm) in 1 ml of TES/NaCl were hybridized for 72 h at 65°C with the indicated linearized

plasmid DNAs (7.5 μg) bound to nitrocellulose filters. The plasmids used in these experiments were $PLA_2$ (a full-length Type II $PLA_2$ cDNA cloned into pGEM3Z), IL-6 (a 601 bp murine cDNA probe cloned into pXM, a gift of Genetics Institute), pRGAPDH-1 (a 1.3 kb rat glyceraldehyde-3-phosphate dehydrogenase [GAPDH]cDNA cloned into pBR322), and a control plasmid, pBR322. After hybridization, the filters were washed twice in 2 × SSC at room temperature, 3 times in 2 × SSC at 65°C for 30 min, and then incubated with RNase A (10 μg/ml) at 37°C for 30 min. The filters were washed subsequently in 2 × SSC/0.2% SDS at 65°C for 30 min, rinsed in 2 × SSC, and exposed for autoradiography. Hybridized nuclear [$^{32}$P]$PLA_2$ and [$^{32}$P]-GAPDH abundance transcript was quantitated using a scanning densitometer (Scan Maker, Microtek, Torrance, CA) and appropriate software (Adobe Photshop, Adobe Systems, Mountain View, CA; and Scan Analysis Biosoft, Cambridge, UK) and corrected for backgrounds (counts hybridized to pBR322 or α-amanitin-insensitive counts hybridized to plasmid). $PLA_2$ transcription rates were determined from the changes in $PLA_2$/GAPDH mRNA ratios assessed under control and IL-1-stimulated conditions.

*Message stability assay.* Mesangial cells were incubated in 5% FBS media in the presence of rIL-1 (10 ng/ml) for 24 h, the time of maximal $PLA_2$ mRNA induction by IL-1. Actinomycin D (5 μg/ml) was added to some dishes. Since certain mRNAs are stabilized in the presence of IL-1 (19), media containing IL-1 was removed from some cells before the addition of actinomycin D. Cells from each experimental protocol were harvested at the indicated times. Total cellular RNA was prepared and analyzed by Northern blotting for $PLA_2$ mRNA expression. In some experiments, mesangial cells were stimulated with IL-1 in the absence of serum. However the presence or absence of serum had no independent effect on $PLA_2$ mRNA turnover, and results from both experimental conditions have been pooled. In some experiments, the blots were rehybridized with the α-tubulin probe, and $T_{1/2}$ for tubulin mRNA was determined from a first-order decay plot.

## Results

*Proinflammatory cytokines induce the expression of a nonpancreatic $PLA_2$ in mesangial cells.* We first characterized the induction of $PLA_2$ mRNA and enzyme expression by rIL-1. As shown in Fig. 1, mesangial cells incubated in 5% FBS in the presence of rIL-1 (10 ng/ml) express a single 1.0 kb $PLA_2$ mRNA transcript (Fig. 1 *A*). The $PLA_2$ transcript was detectable by 6 h of incubation with rIL-1. Transcript abundance increased with time and reached a plateau after 16 h (Fig. 1 *B*). In 24 hour incubations, rIL-1 induced a dose-dependent, coordinate increase in $PLA_2$ mRNA and activity (Fig. 1 *C*, Table I). The threshold concentration for rIL-1 stimulated $PLA_2$ expression was 1.0 ng/ml. The rIL-1-induced increase steady state $PLA_2$ mRNA was not affected by coincubation with cycloheximide for two (not shown), four (not shown), or 24 h (Fig. 2 *A*). Neither message abundance nor the kinetics of $PLA_2$ mRNA induction were altered, suggesting that this transcript is not superinduced or rapidly degraded. Inclusion of actinomycin D did abolish the rIL-1 induced gene expression (Fig. 2 *B*), consistent with its effect on the $PLA_2$ activity measured previously in acid extracts (9). Taken together, these data suggest that the rIL-1 stimulated $PLA_2$ activity requires de novo RNA synthesis that results in the coordinate synthesis and secretion of enzyme.

We next determined whether the IL-1-induced increase in $PLA_2$ mRNA levels was in fact due to enhanced transcription, increased mRNA stability or both. Fig. 3 *A* shows a representative run-off transcription analysis which demonstrates that rIL-1 increased the rate of $PLA_2$ mRNA transcription. In three separate experiments, analysis of $PLA_2$ nuclear transcript abundance, corrected for background and normalized for changes in GAPDH transcripts, indicated that $PLA_2$ transcription rate increased 219±18% in cells stimulated with IL-1 for 15 h compared to cell incubated with vehicle. The rIL-1-stimulated increase in $PLA_2$ transcription rate was suppressed by concentrations of α-amanitin that specifically inhibit polymerase II activity (not shown). We also examined whether IL-1 also had an effect on $PLA_2$ RNA turnover (Fig. 3 *B*). In the presence of actinomycin D, $PLA_2$ mRNA degradation is exceeding slow, a finding which was unaffected by the presence of IL-1 in the incubation medium. In contrast, the half-life of tubulin transcripts in the same cells was ~3 h, a decay rate similar to that reported for this mRNA in other cell lines (20).

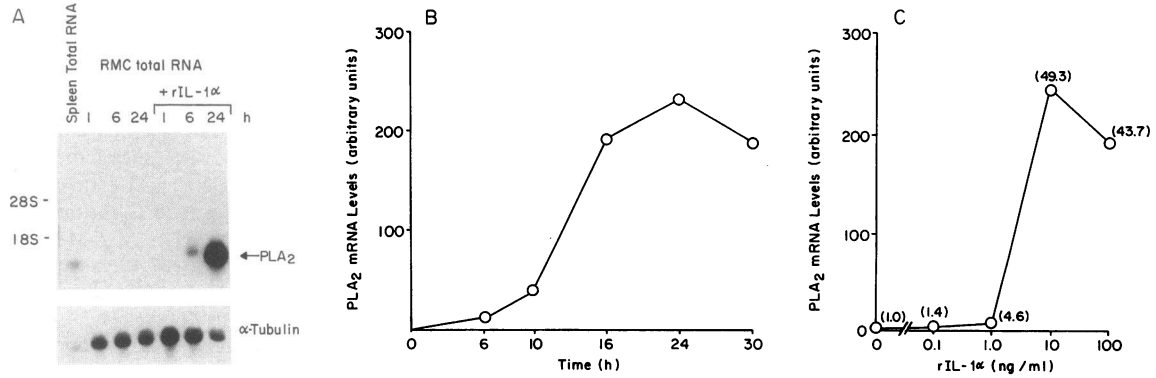



*Figure 1.* rIL-1 induces $sPLA_2$ mRNA expression. Each experiment is representative of two to three replicates. (*A*) Northern Analysis. Mesangial cells were incubated with rIL-1 (10 ng/ml) or vehicle for the indicated times. Total cellular RNA (20 μg) was analyzed for $PLA_2$ by Northern analysis as described in Methods and demonstrated a 1.0 kb species only in cytokine-stimulated cells. The filter was rehybridized with chicken α-tubulin (1.2 kb) to demonstrate the integrity of the RNA, the equivalence of loading, and the specificity of mRNA induction. Spleen RNA was also analyzed as a positive control and demonstrates a 0.8 kb $PLA_2$ transcript. (*B*) Time course for rIL-1 induced $PLA_2$ expression. Mesangial cells were incubated as stated above for the times indicated in the figure. Total cellular RNA (20 μg) was analyzed for $PLA_2$ message using RNA–RNA solution hybridization as described in detail in Methods and in Fig. 2. Autoradiograms were quantitated by densitometry and the results presented as total peak area for each time point. (*C*) Dose-dependence of IL-1-induced $PLA_2$ mRNA expression. $PLA_2$ expression at 24 h was analyzed as described for graph *B*. $PLA_2$ activity in the medium was measured as described in Methods and is shown in parenthesis as the relative stimulation above the control value (1.0). Actual control and rIL-1 stimulated mean $PLA_2$ activities are shown in Table I.

*Table I. Secreted $PLA_2$ Activity in Response to the Indicated Agonists*

| Condition | $PLA_2$ activity |
|---|---|
| | *nmol hydrolyzed/h* |
| A. *Cycling MC* | |
| Control | 60±10 |
| IL-$\alpha$ (10 ng/ml) | 1,680±260 |
| IL-$\beta$ (50 ng/ml) | 3,200±110 |
| LPS (10 $\mu$g/mL) | 250±10 |
| IL-2 (1,500 U/ml) | 40±10 |
| Forskolin (10 $\mu$M) | 60±10 |
| Forskolin + rIL-1$\alpha$ | 5,210±900 |
| Dibutyryl cAMP (1 mM) | 50±10 |
| Dibutyryl + rIL-1$\alpha$ | 2,280±40 |
| DiOG (100 $\mu$M) | 70±10 |
| OAG (50 $\mu$g/ml) | 70±10 |
| PA (50 $\mu$g/ml) | 67±10 |
| TPA (0.1 $\mu$M) | 50±10 |
| Dexamethasone (1 $\mu$M) | 50±10 |
| Dexamethasone + rIL-1$\alpha$ | 1,340±15 |
| B. *Serum-Deprived MC* | |
| rIL-1$\alpha$ (10 ng/ml) | 220±30 |
| PDGF (25 ng/ml) + rIL-$\alpha$ | 139±30 |

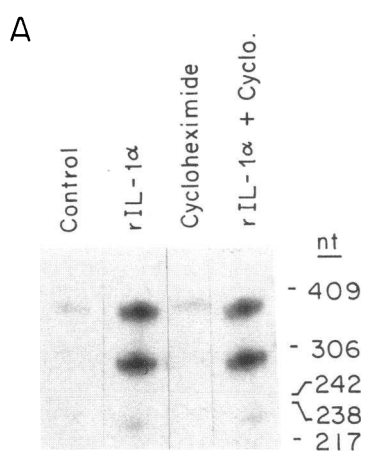
Mesangial cells were incubated for 24 h with the indicated agonist as described in Methods. $PLA_2$ activity was expressed as the total activity present in the media at the end of the incubation period. Results were expressed as the mean±SEM of 2–7 determinations.

These data demonstrate that IL-1-stimulated increases in steady state $PLA_2$ mRNA abundance resulted from a two-to-three-fold increase in the $PLA_2$ transcription rate that is amplified by the prolonged persistence of the transcript. Although the lack of $PLA_2$ mRNA degradation is striking, agonist stimulated accumulation of other mRNAs have been reported to result from a similar mechanism (20).

To better define the potential role of $PLA_2$ in immune-mediated glomerular injury, we next examined the effect of several cytokines, which have been implicated in the pathogenesis of experimental and human glomerulonephritis, on mesangial cell $PLA_2$ expression. The data are shown in Fig. 4. TNF, rIL-1$\alpha$, and rIL-$\beta$ markedly stimulated $PLA_2$ message expression. LPS was less potent while IL-2 had no effect. As indicated, enzyme activity released into the medium (Fig. 4, Table I) was coordinate with mRNA induction. In addition, neither PDGF (see below) nor epidermal growth factor (not shown) stimulated mesangial cell $PLA_2$ expression.

*Forskolin and dibutyryl cAMP potentiate but do not mediate rIL-1-stimulated $PLA_2$ expression.* We are interested in defining the pathways used by IL-1 in mesangial cells to activate a change in cellular function. IL-1 induces cAMP accumulation in some cells, and $PLA_2$ activity has been reported to be stimulated by cAMP-elevating agents in vascular smooth muscle cells (21), astrocytes (22), and rat mesangial cells (23). We therefore examined the effect of forskolin and dibutyryl cAMP on $PLA_2$ gene expression and $PLA_2$ activity. As shown in Fig. 5 *A*, addition of forskolin (10 $\mu$M) significantly enhanced IL-1-induced $PLA_2$ message expression by nearly fourfold (360±41% stimulation, mean±SEM, $n = 3$ individual experiments, $P < 0.02$). Similar results were obtained for a permeable cAMP analog, dibutyryl cAMP (1 mM) (Fig. 5 *B*). The amount of $PLA_2$ activity in the medium reflected the degree of mRNA expression (Fig. 5, Table I). In contrast, neither forskolin nor dibutyryl cAMP alone induced $PLA_2$ gene expression (Fig. 5) or release of enzyme activity (Table I). The inactive forskolin analog 1, 9-dideoxyforskolin, (10 $\mu$M), butyrate (0.2 mM), and dibutyryl cGMP (1 mM) also failed to potentiate the IL-1 response (data not shown). Taken together, these results suggest that activation of protein kinase A potentiates but does not mimic IL-1-stimulated mesangial cell $PLA_2$ expression.

*Diradylglycerols, phosphatidic acid, and phorbol ester do not mimic or alter rIL-1-induced $PLA_2$ expression.* We and others have shown that several lipid signals are rapidly formed in IL-1-stimulated mesangial cells, including 1,2-diacylglycerol and phosphatidic acid (10, 11). As shown in Fig. 6 *A*, the



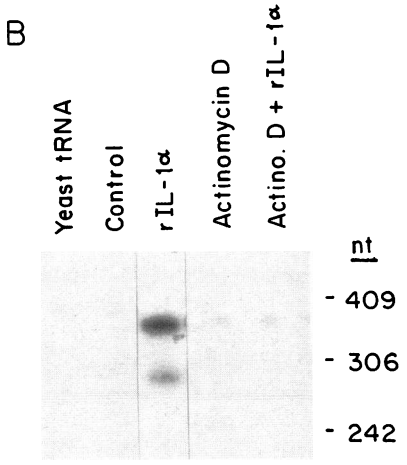




*Figure 2.* Effect of protein (diagram *A*) and RNA synthesis (diagram *B*) inhibitors on IL-1-stimulated $PLA_2$ message expression. Mesangial cells were incubated in the presence of rIL-1 (10 ng/ml) or vehicle for 24 h. Cycloheximide (1 $\mu$g/ml) or actinomycin D (0.2 $\mu$g/ml) was added concurrently to some dishes with or without rIL-1. Total RNA was analyzed for $PLA_2$ transcripts by RNA–RNA solution hybridization as described in Methods. A [$^{32}$P]riboprobe was synthesized from a linearized DNA template that contained the cDNA sequence (bp 381–758) for rat splenic nonpancreatic (type II) $PLA_2$. This $PLA_2$ mRNA contains a heterologous nucleotide (nt 461) which generates the indicated protected fragments shown (378 nt and 297 nt). Markers were [$^{32}$P]labeled fragments of a MSP I digest pBR322. The figure is representative of duplicate experiments.

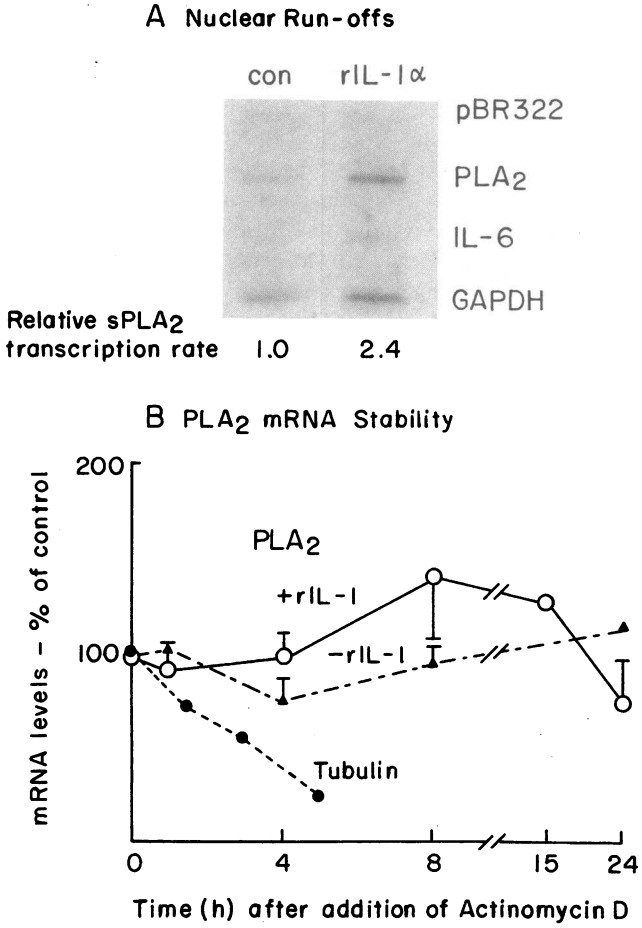



*Figure 3.* Effect of rIL-1 on $PLA_2$ mRNA transcription (*A*) and stability (*B*). (*A*) Mesangial cells were incubated with RPMI media alone or with rIL-1 (10 ng/ml) for 15 h. Nuclei were isolated and a run-off transcription analysis was performed as described in Methods. Densitometric scans of the nascent transcripts hybridized to each plasmid were obtained, and the $PLA_2$ transcription rate was quantified as described in Methods. This diagram is representative of three individual experiments. (*B*) The effect of rIL-1 on $PLA_2$ mRNA turnover was assessed by Northern analysis. Mesangial cells were incubated in the presence of IL-1 (10 ng/ml) for 24 h. Actinomycin D (5 μg/ml) was added and the cells harvested at the indicated times (*curve marked* "+ IL-1"). In some experiments, IL-1 was removed and fresh media containing actinomycin D was added (*curve marked* "–IL-1"). Total cellular RNA (20 μg) was analyzed for $PLA_2$ mRNA abundance by Northern analysis. α-tubulin mRNA levels were determined also and a representative decay curve is shown. $PLA_2$ and tubulin mRNA levels were quantified as described in Methods and are expressed as percent of transcript abundance prior to the addition of actinomycin D (0 time value). $PLA_2$ mRNA levels are the mean±SEM for 2–5 experiments.

addition of diacylglycerol analogs, DiOG (100 μM) or OAG (50 μg/ml), did not stimulate $PLA_2$ gene expression or enhance phospholipase $A_2$ activity in the medium (Table I). Phosphatidic acid (50 μg/ml) also failed to stimulate $PLA_2$ expression (Fig. 6 *B*). The protein kinase C activator TPA also did not increase mesangial cell $PLA_2$ mRNA or enzyme activity (Fig. 6 *B* and Table I) or alter rIL-1-induced $PLA_2$ synthesis when it was coincubated with the cytokine (data not shown). These observations indicate that the effects of IL-1 on the mesangial cell are not mediated or modified by the activation of protein kinase C. Consistent with this supposition, we have also shown by two-dimensional gel electrophoresis that rIL-1 does not increase the phosphorylation state of the putative 80 kD substrate of protein kinase C (data not shown).

*Serum modulates IL-1-stimulated $PLA_2$ expression.* Serum markedly alters mesangial cell $PGE_2$ synthesis in response to rIL-1 (9), in part due to the action of serum on prostaglandin endoperoxide synthesis expression (24). We have now assessed the role of serum on IL-1 stimulation of $PLA_2$ gene expression and activity. As shown in Fig. 7 *A*, steady state $PLA_2$ message levels in cells incubated with rIL-1 in the absence of serum were two-fold higher than those observed with rIL-1 in serum-treated cells (209%±27%, mean±SEM, $n = 3$ individual experiments, $P \leq 0.05$). $PLA_2$ activity measured in acid-extracts from serum-stimulated and serum-deprived, rIL-1-stimulated mesangial cells was concordant with mRNA expression (Fig. 7 *B*). In cells coincubated with 5% FBS and rIL-1, $PLA_2$ activity was 220±38 fmol/μg per hr compared to rIL-1 stimulated activity in the absence of serum of 590±28 fmol/μg per hr, (mean±SEM). The presence or absence of serum alone in did not significantly alter $PLA_2$ activity in acid extracts of mesangial cells. To assess whether the effect of serum on genes induced by rIL-1 was specific for $PLA_2$, we also studied IL-1-stimulated IL-6 gene expression in the presence and absence of FBS. In contrast to its inhibitory action on $PLA_2$ expression, serum did not inhibit IL-6 steady state mRNA levels (data not shown). The inhibitory effect of serum on $PLA_2$ message appears to be specific and not due to an alteration in IL-1 bioactivity, receptor expression or responsiveness of the cells to cytokine.

We next examined potential mechanisms by which serum could regulate IL-1-stimulated $PLA_2$ expression. Serum contains a number of biologically active compounds, including hormones and growth factors. The synthetic glucocorticoid dexamethasone has been shown previously to inhibit $PLA_2$ activity and the expression of other IL-1-stimulated mRNAs and proteins (25). However, coincubation of mesangial cells with rIL-1 and dexamethasone did not significantly change $PLA_2$ mRNA abundance or activity when compared to cells exposed to rIL-1 alone (Fig. 8 *A* and Table I).

Several growth factors in serum activate receptor protein tyrosine kinases. The effect of the tyrosine kinase inhibitor, genistein, on cytokine-stimulated mesangial cell $PLA_2$ mRNA expression is shown in Fig. 8 *B*. Genistein (10 μg/ml) markedly increased rIL-1-stimulated message accumulation in serum-stimulated RMC compared to vehicle (444±12% stimulation, mean±SEM, duplicate experiments). Genistein at this concentration had no effect on cell viability as assessed by an assay of mitochondrial respiration (9). The growth factor PDGF is an abundant constituent of serum, activates a receptor tyrosine kinase, and is a potent mesangial cell mitogen and a mediator of glomerular injury. PDGF (25 ng/ml) alone did not stimulate $PLA_2$ mRNA synthesis or enzyme activity in serum-deprived cells but mimicked the effect of 5% FBS on IL-1-induced $PLA_2$. When cells were coincubated with PDGF and IL-1, $PLA_2$ transcript abundance was reduced by 72±8%, (mean±SEM, duplicate experiments). PDGF also attenuated the IL-1-stimulated $PLA_2$ activity released in the media by 44%±9 (Table I). Taken together, these data suggest that IL-1-stimulated $PLA_2$ expression can be regulated by growth factor activation of receptor tyrosine kinases.

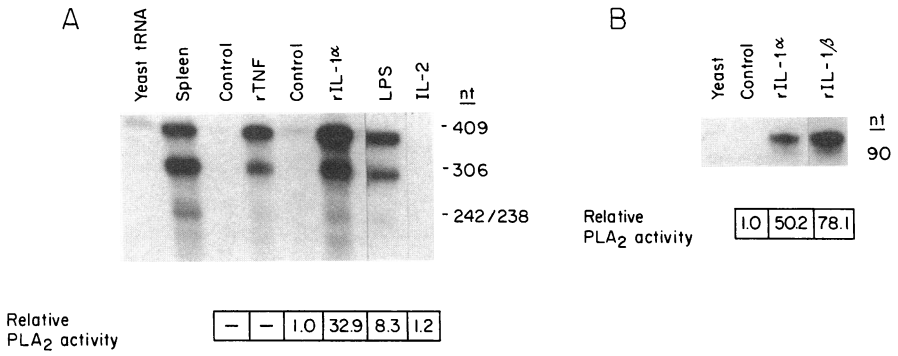



*Figure 4.* Effect of cytokines and LPS on $PLA_2$ message expression and activity. Each panel is representative of at least duplicate experiments. (*A*) Total RNA was isolated from mesangial cells incubated with TNF (50 ng/ml), rIL-1$\alpha$ (10 ng/ml), rIL-1$\beta$ (50 ng/ml), LPS (10 $\mu$g/ml), or IL-2 (1,500 U/ml) for 24 h. $PLA_2$ message was quantitated using RNA–RNA solution hybridization using the probe described in Fig. 2. (*B*) Hybridization conditions were identical to *A*, except that the [$^{32}$P]cRNA probe used was transcribed from a cDNA template that contained the sequence (bp 666–759) of rat spleen nonpancreatic (type II) $PLA_2$ (see Methods). $PLA_2$ activity released into the medium in response to the indicated stimulus was quantitated and normalized as described in Fig. 1, and is expressed under the appropriate lane in each panel. Actual $PLA_2$ activities for the condition in *A* and *B* are shown in Table I.

## Discussion

IL-1 and TNF activate mesangial cells to express new structural and functional characteristics, a process which involves specific changes in gene activity in the nucleus. We have studied regulation of a nonpancreatic (Type II) $PLA_2$ to understand the intracellular pathways engaged in cytokine-activated mesangial cells. $PLA_2$ expression provides a reasonable model system for defining mechanisms of transcriptional induction by IL-1 in mesangial cells, since we have shown this gene is transcriptionally regulated by IL-1, and that it is not detectable in quiescent, serum-stimulated, or growth factor-activated cells.

The basis for the specificity in gene induction by particular polypeptide ligands is poorly understood. In this study, we have used second messenger mimics to explore the intracellular pathways used by IL-1 to stimulate gene transcription. Our data show that protein kinase C activation (this paper), cAMP accumulation (this paper, reference 14), or rapid changes in cytosolic $Ca^{2+}$ or $pH_i$ (10) are not required for IL-1-directed $PLA_2$ gene expression and suggest that other signaling pathways are engaged directly by IL-1 to induce $PLA_2$ mRNA expression. Since $PLA_2$ gene expression is cycloheximide insensitive, we believe these signals ultimately activate transcription factor(s) by posttranslational modifications.

Several mechanisms can be proposed to describe how IL-1–IL-1-receptor interactions directly alter gene activity in the nucleus. Traditional models of transmembrane signaling propose that a limited number of second messengers convey the information generated by a ligand–receptor interaction. Using this model in conjunction with published information describing IL-1-activated transmembrane signaling pathways (2, 10–14), we would suggest that IL-1 alters the phosphorylation state of a transcriptional factor or transcription factor complex by activating a kinase cascade. IL-1 can change protein phosphorylation patterns by activating extracellular signal-related kinase/mitogen-activated peptide kinase (ERK/MAP kinase) (26, 27) or by inhibiting phosphatase activity (28). Recent data suggest phosphorylation state can regulate transcriptional factor activity. Both c-jun and CREB are positively regulated by phosphorylation (29, 30). Translocation of the rel/k$\beta$ proteins

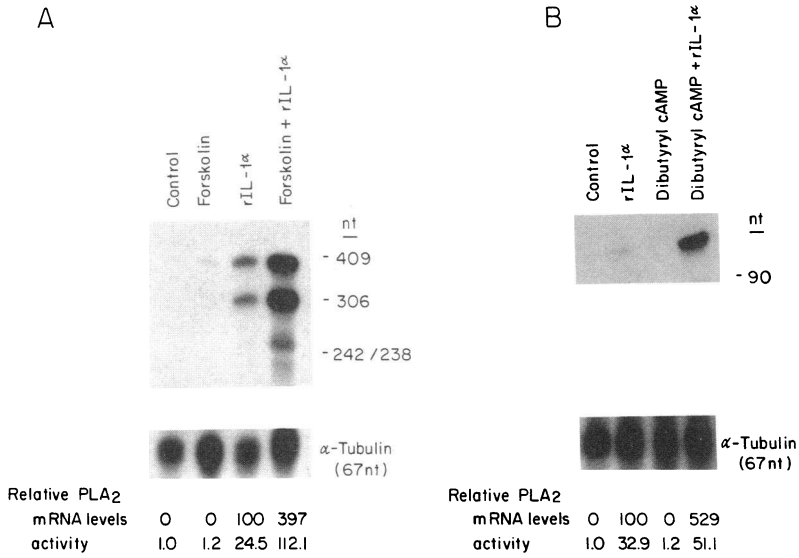



*Figure 5.* Forskolin (*A*) and dibutyryl cAMP (*B*) enhance rIL-1-stimulated $PLA_2$ mRNA expression. Mesangial cells were incubated for 24 h with forskolin (10 $\mu$M) or dibutyryl cAMP (1 mM) with or without rIL-1 (10 ng/ml). The data presented in *A* is representative of three separate experiments and that in *B* is representative of duplicate experiments. (*A*) Total mRNA (15 $\mu$g) was analyzed by solution hybridization for $PLA_2$ message expression using the probe described in Fig. 2. The samples were concurrently hybridized with a [$^{32}$P]probe for chicken $\alpha$-tubulin. The signal intensities of the protected fragments for each mRNA species were quantitated by densitometry, and the $PLA_2$ mRNA expression was corrected for changes in the tubulin transcript abundance. Relative mesangial cell $PLA_2$ mRNA expression after rIL-1-stimulation was arbitrarily defined as 100 units. Released $PLA_2$ activity was quantified and expressed as described in Fig. 1. (*B*) Total mRNA (15 $\mu$g) was analyzed for $PLA_2$ message and quantified as described in *A*, except the [$^{32}$P]$PLA_2$ probe described in Fig. 3 *B* was used. Relative $PLA_2$ activity is presented as described in Fig. 1. Actual $PLA_2$ activities for the conditions shown in *A* and *B* are presented in Table I.

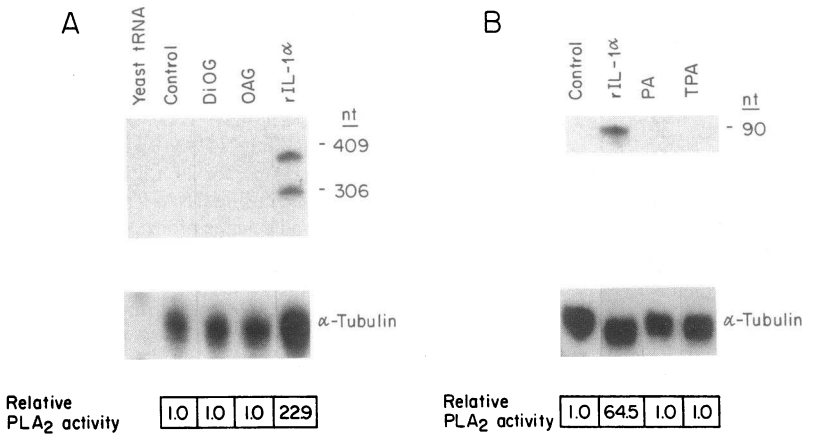



*Figure 6.* The effect of diradyglycerols, phorbol ester, and phosphatidic acid on mesangial cell $PLA_2$ mRNA expression. (*A*) Mesangial cells were incubated for 24 h in medium in the presence or absence of DiOG (100 $\mu$M), OAG (50 $\mu$g/ml) for 24 h. Total RNA (15 $\mu$g) was analyzed for $PLA_2$ message, using the probe described in Fig. 2, and tubulin mRNA. The panel is representative of two experiments. (*B*) Mesangial cells were incubated for 24 h with rIL-1 (10 ng/ml), PA (50 $\mu$g/ml) or TPA (0.1 $\mu$M) and analyzed for $PLA_2$ and tubulin mRNA expression as described in *A*, except the probe described in Fig. 3 *B* was used. The figure is representative of two separate experiments. In both *A* and *B* relative $PLA_2$ activity in the medium is presented as previously described in Fig. 1. Actual $PLA_2$ activities for the conditions shown in *A* and *B* are in Table I.

to the nucleus requires phosphorylation of associated inhibitory proteins, which tether them in the cytosol (31). In this model, specificity of gene induction by IL-1 results from unique interactions between activated transcription factors.

Recent work, which describes the pathways used by interferon-$\alpha/\beta$ to induce gene expression, suggests an alternative model (32–34). Treatment of cells with interferon immediately phosphorylates three proteins, which comprise a unique transcriptional activator that translocates to the nucleus to induce a specific gene set. These data, which suggest the existence of many ligand-specific, direct signaling pathways, would predict that binding of IL-1 to its receptor catalyzes a posttranscriptional modification of a specific, latent cytoplasmic transcription factor(s). This latter model is consistent with our data demonstrating the inability of second messenger mimics to induce $PLA_2$ mRNA expression. However, neither IL-1-specific transcriptional proteins nor specific IL-1 responsive DNA elements have been identified.

In the context of other published studies (21, 22, 35), our findings with second messenger molecules demonstrate that Type II $PLA_2$ expression is regulated in a cell-specific manner. Raising cytosolic cAMP levels potentiates IL-1-induced $PLA_2$ mRNA enzyme activity in all cell types studied (21, 22), but the adenylate cyclase activator forskolin in the absence of cytokine induces $PLA_2$ synthesis only in vascular smooth muscle cells (21). The protein kinase activator TPA stimulates astrocytes to synthesize and release $PLA_2$ (22), but fails to increase either $PLA_2$ mRNA abundance or enzyme activity in the mesangial cell and vascular smooth muscle cell (21). Finally, cycloheximide induces $PLA_2$ mRNA in smooth muscle cells, suggesting the existence of short-lived negative regulators (35), but does not superinduce $PLA_2$ transcript expression in mesangial cells. The regulation of $PLA_2$ synthesis appears to be complex and highly dependent on the target cell type and the stimulus employed. Cell specific expression of the gene appears to result, at least in part, from unique interactions between signaling pathways.

Other polypeptide ligands present in the glomerular microenvironment may modulate induction of $PLA_2$ expression by IL-1. In vitro, serum negatively regulates IL-1-stimulated

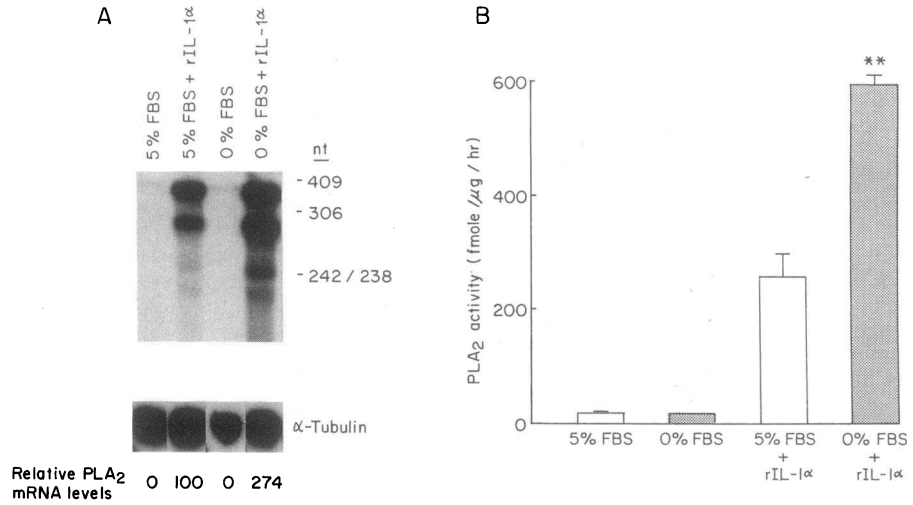



*Figure 7.* Suppression of rIL-1-stimulated $PLA_2$ expression by serum. (*A*) Mesangial cells were incubated for 24 h in the presence or absence of rIL-1 (10 ng/ml) in RPMI media with or without 5% FBS. Total RNA (15 $\mu$g) was analyzed by RNA–RNA solution hybridization using the [$^{32}$P]-probe described in Fig. 2. $PLA_2$ mRNA abundance were corrected for changes in tubulin mRNA expression as previously described in Fig. 4. The effect of rIL-1 in the presence of 5% FBS is arbitrarily defined as 100 units. This panel is representative of three separate experiments. (*B*) $PLA_2$ activity in acid extracts of mesangial cells. The cells were cultured for 24 h in the presence of rIL-1 (10 ng/ml) with or without 5% FBS. Cell homogenates were acid-extracted and assayed for $PLA_2$ activity as described in Methods. Data are expressed as mean±SEM, for 2–5 individual experiments. $^{**}P < 0.005$, compared to cells incubated with rIL-1 in the presence of 5% FBS.

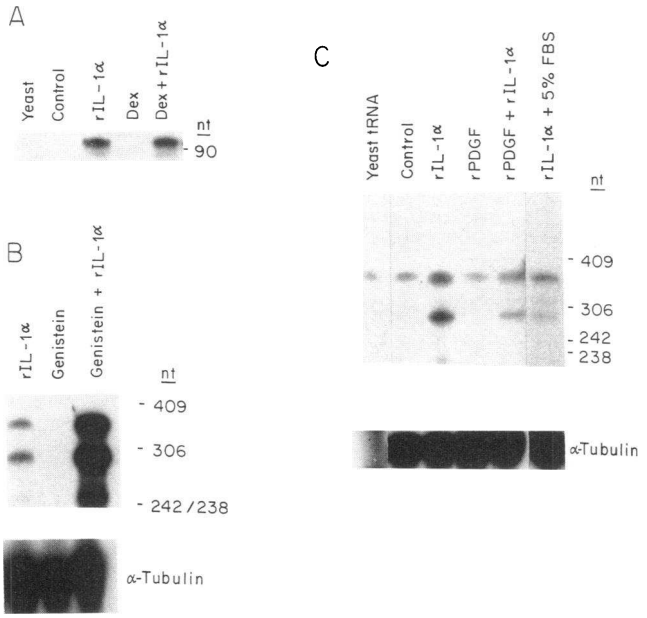



*Figure 8.* Regulation of rIL-1 stimulated $PLA_2$ expression by serum components. Each panel is representative of at least two separate experiments. (*A*) Cells were cultured for 24 h in the absence of serum with dexamethasone (Dex, 1 μM) or vehicle in the presence or absence of rIL-1 (10 ng/ml). Mesangial cell $PLA_2$ mRNA expression was analyzed using the probe described in Fig. 3 *B*. (*B*) Mesangial cells were incubated in 5% FBS, and treated with genistein (10 μg/ml) or vehicle 30 min before the addition of rIL-1 (10 ng/ml) or vehicle. Parallel studies confirmed this concentration of genestein blocked growth factor activated receptor tyrosine kinase activity. Both PDGF-stimulated tyrosine phosphorylation, as determined by immunoblotting, and mesangial cell mitogenesis, as assessed by [$^3$H]-thymidine incorporation and cell counts, were inhibited. Cellular RNA was harvested after 24 h and $PLA_2$ and tubulin message were analyzed as previously described. (*C*) Cells were serum-restricted for 3 d then incubated with PDGF (25 ng/ml), rIL-1 (10 ng/ml), or both for 24 h. Total RNA was isolated and analyzed for $PLA_2$ and tubulin message as previously described.

mesangial cell $PLA_2$ mRNA expression and enzyme activity but not IL-6 mRNA expression. This specificity in the effect of serum suggests the mechanism does not involve an inhibitor of IL-1 present in FBS (36) or the down-regulation of IL-1 receptors by chronic protein kinase C activation (37). Genistein, a relatively selective inhibitor of tyrosine kinase activity at the concentration employed, however, enhances IL-1-stimulated $PLA_2$ mRNA expression in serum-stimulated cells. Growth factor-activated receptor tyrosine kinases may mediate the effect of serum on $PLA_2$ mRNA abundance. Consistent with this hypothesis, coincubation of mesangial cells with PDGF and IL-1 reproduces the effect of serum on $PLA_2$ expression. Although PDGF also stimulates protein kinase C activity, neither TPA nor diacylglycerol analogues reduce IL-1-stimulated $PLA_2$ mRNA formation. Two recent studies have reported also that PDGF reduces $PLA_2$ protein synthesis (38, 39). Our results demonstrate this inhibition occurs at the level of mRNA abundance. Growth factors such as PDGF may counter-regulate the proinflammatory actions of cytokines. PDGF inhibits cytokine-stimulated synthesis of the complement protein Factor B, and potentiates IL-1-stimulated production of metalloproteinases in human fibroblasts through changes in mRNA abundance (40, 41). Secretory Group II $PLA_2$ promotes inflammatory injury (42). Growth factor-dependent, down-regulation of $PLA_2$ secretion may act to limit tissue destruction and shift the local balance to favor healing and tissue repair.

Recently a high molecular weight intracellular $PLA_2$ ($cPLA_2$), that is mechanistically and biochemically distinct from the Type II 14-kD $PLA_2$ we have studied, has been described in the mesangial cell (42, 43). We and others have demonstrated that within minutes IL-1 activates $cPLA_2$ by phosphorylation[2] (44, 45). However, the precise function of each isoform in arachidonate metabolism and mesangial cell function and glomerular inflammation remains to be defined. Some, but not all studies, have suggested that activation of both isoforms results in arachidonic acid hydrolysis (42, 45–47). The 14-kD, secreted $PLA_2$ may be targeted to the cell membrane to hydrolyze phospholipids, an action that fails to irreversibly injure the cell (46, 47). A regulatory mechanism must prevent lethal cell injury to allow the low molecular weight $PLA_2$s to alter cell function independently of membrane disruption.

Both in vitro and in vivo studies suggest the mesangial cell expresses distinct phenotypes in response to changes in the glomerular microenvironment. Regulation of the Type II $PLA_2$ gene presents a useful model for understanding how regulatory proteins and intracellular signaling pathways fit together to determine mesangial cell fate. Future work on this model system hopefully will discriminate the distinctive signaling pathways used by the mesangial cell for specialized responses to cytokines and provide insight in kidney-specific mechanisms of gene expression.

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