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Modulation of Cytokine-Induced Expression of Secretory Phospholipase A_2-type IIA by Protein Kinase C in Rat Renal Mesangial Cells

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ABSTRACT. Renal mesangial cells express the 14 kDa secretory phospholipase A_2-type IIA ($sPLA_2$-IIA) in response to interleukin-1β (IL-1β). In order to understand the regulation of cytokine-induced $sPLA_2$-IIA induction in more detail, we investigated whether phorbol ester-activated protein kinase C (PKC) has an influence on the IL-1β-induced expression of $sPLA_2$-IIA. We found that treatment of mesangial cells with the biologically active phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate inhibited IL-1β induction of $sPLA_2$-IIA mRNA, protein, and activity, whereas the inactive compound 4α-phorbol 12,13-didecanoate was without effect. An 8-hr pretreatment with PMA, which led to down-regulation of PKC-α and -δ isoenzymes, still inhibited $sPLA_2$-IIA induction. Only after down-regulation of PKC-ε isoenzyme by 24-hr preincubation with PMA were we able to reconstitute the IL-1β-induced $sPLA_2$-IIA expression. Thrombin as a physiological activator of PKC in mesangial cells exerted similar effects as PMA and inhibited $sPLA_2$-IIA expression. The selective PKC inhibitor calphostin C potentiated IL-1β induction of $sPLA_2$-IIA mRNA levels and partially reconstituted the thrombin-induced inhibition of $sPLA_2$-IIA mRNA and activity. These data show that IL-1β induction of $sPLA_2$-IIA can be modulated by PKC and that the ε-isoenzyme of the PKC family is the most likely candidate mediating the suppression of cytokine-induced $sPLA_2$-IIA expression in mesangial cells. BIOCHEM PHARMACOL **58**;11:1751–1758, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. interleukin-1β; mesangial cells; phorbol ester; phospholipase A_2; PKC; thrombin

$sPLA_2$-IIA‖ belongs to a highly conserved family of enzymes with lipolytic activity which are secreted from mammalian cells and are found in soluble form in high amounts at inflammatory sites of diseases such as rheumatoid arthritis or psoriasis. In glomerular mesangial cells, which are recognized as important target and effector cells in the pathogenesis of renal diseases, $sPLA_2$-IIA is thought to participate in the initiation and propagation of inflammatory reactions in the kidney. Several investigations have shown that proinflammatory cytokines such as IL-1β and tumor necrosis factor α or cyclic AMP-elevating agents such as forskolin up-regulate the expression of the $sPLA_2$-IIA (for review see [1, 2]). Under these conditions, the enzyme seems to support and promote the action of $cPLA_2$ by releasing arachidonic acid and thereby directly or indirectly increasing the production of inflammatory lipid mediators such as prostaglandin E_2 [3]. Recently, it was shown that exogenously added $sPLA_2$ from snake venom or human $sPLA_2$-IIA activated the Raf-1/mitogen-activated protein kinase cascade and PKC to coordinate cross-communication with $cPLA_2$ and subsequent eicosanoid synthesis in mesangial cells [4]. This cell type expresses four PKC isoenzymes, PKC-α, -δ, -ε, and -ζ [5–7], of which PKC-ε was shown to mediate hormone-stimulated $cPLA_2$ activation [5]. However, up to now a long-term regulatory role of PKC isoenzymes in cytokine-induced expression of $cPLA_2$ or $sPLA_2$-IIA had not been investigated. Therefore, we studied the effects of the biologically active phorbol esters PMA and PDBu and the inactive PDD on the IL-1β-induced expression of $sPLA_2$-IIA, the secretion of the protein as well as its enzymatic activity. As thrombin is a physiological activator of PKC, we investigated its effects on cytokine-induced $sPLA_2$-IIA expression in mesangial cells. Thrombin has been shown to stimulate phosphoinositide turnover and to trigger subsequent Ca^{2+} mobilization and PKC activation in mesangial cells [8, 9]. Moreover, we used the specific PKC inhibitor calphostin C in order to

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‖ *Abbreviations:* AP-1/2, activator protein-1/2; $cPLA_2$, cytosolic phospholipase A_2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IRAKs, interleukin-1 receptor-associated kinases; IL-1β, interleukin-1β; IL-1RI, interleukin-1 type I receptor; NFκB, nuclear factor-κB; PDBu, phorbol 12,13-dibutyrate; PDD, phorbol 12,13-didecanoate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; and $sPLA_2$-IIA, secretory phospholipase A_2-type IIA.

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investigate the regulation of $sPLA_2$-IIA expression in mesangial cells. Our data indicate that PKC-ε is a main candidate acting as a modulator of $sPLA_2$-IIA expression in mesangial cells.

MATERIALS AND METHODS

Materials

Recombinant IL-1β was supplied by Dr. C. Rordorf, Novartis Pharma Inc. α-Thrombin (specific clotting 3488.6 U/mg) was a gift from Dr. Fenton II, Albany, NY. [1-^{14}C]Oleic acid and [^{32}P]dCTP (110 TBq/mmol) were from Amersham-Buchler. The cDNA clone coding for GAPDH was a gift from Dr. Karl-Friedrich Beck, Institute of Pharmacology, University of Frankfurt. Nylon membranes (Gene Screen) were purchased from NEN Life Science. The phorbol esters were purchased from Sigma. Calphostin C was from Calbiochem. All cell culture media and nutrients were from GIBCO BRL, and all other chemicals used were from either Merck or Fluka.

Cell Culture

Rat renal mesangial cells were cultivated as described previously [10]. The cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and bovine insulin (0.66 units/mL). Twenty-four hours prior to stimulation and during the experiments, cells were incubated in Dulbecco's modified Essential medium containing 0.1 mg/mL fatty acid-free BSA.

Phospholipase A_2 Assay

$sPLA_2$-IIA activity in the supernatant of mesangial cell cultures was determined with [1-^{14}C]oleate-labelled *Escherichia coli* as substrate as described previously [11]. Assay mixtures (750 μL) contained 100 mM Tris/HCl (pH 7.0), 1 mM $CaCl_2$, [1-^{14}C]oleate-labelled *E. coli* (≈5000 cpm), and the enzyme-containing supernatants of the cell cultures (about 15–20 μL) at a dilution producing 5% substrate hydrolysis. Reaction mixtures were incubated for 1 hr at 37° in a thermomixer. The reaction was stopped by the addition of 50 μL 1 mM EGTA/1 N HCl and 800 μL ethylacetate. After extraction of the lipids the organic phase was dried in a vacuum concentrator. The lipids were then dissolved in 50 μL ethylacetate and separated by thin layer chromatography on silicagel G 60 plates using the organic phase of ethylacetate/isooctane/acetic acid/water (110/50/20/100 by vol.) as a solvent system. The detection and quantification of the separated [1-^{14}C]oleic acid was performed with a BAS 1500 phosphorimager from Fuji. The $sPLA_2$-IIA activity is defined as image quants counted from the spots corresponding to [^{14}C]oleic acid.

Northern Blot Analysis

Confluent mesangial cells were cultured in 100-mm diameter culture dishes. After stimulation, cells were washed twice with PBS and harvested using a rubber policeman. Total cellular RNA was extracted from the cell pellets using the guanidinium isothiocyanate/phenol/chloroform method. Samples of 10 μg of RNA were separated on 1.4% agarose/formaldehyde gels and transferred to a gene screen membrane. After UV cross-linking and prehybridization for 4 hr, the filters were hybridized for 16 hr at 42° to a [^{32}P]labelled cDNA insert from $sPLA_2$-IIA [12]. DNA probes were radioactively labelled with [α-^{32}P]dCTP by random priming. Finally, the filters were washed twice with 2× sodium chloride/sodium citrate/0.1% SDS for 2 × 20 min and several times at 65° with 0.2× sodium chloride/sodium citrate/1% SDS. The signal was detected with a BAS 1500 phosphorimager (see above). To correct for variations in the amount of RNA, the $sPLA_2$-IIA probe was stripped and the blots were rehybridized to the [α-^{32}P]dCTP-labelled cDNA insert for GAPDH. For quantification the signals of the filters were scanned densitometrically. The signal density of each of the RNA samples hybridized to the $sPLA_2$-IIA probe was divided by that hybridized to the GAPDH probe.

Western Blot Analysis

$sPLA_2$-IIA protein secretion by the cells was assayed by precipitating 1 mL of the culture supernatant with 400 μL of 20% trichloroacetic acid. SDS-PAGE using a 15% polyacrylamide gel was performed under non-reducing conditions according to Laemmli [13]. The proteins were transferred to polyvinylidene difluoride membranes for 2 hr at 2 mA/cm^2. Non-specific binding was blocked with 0.1% (w/v) milk powder in PBS for 1 hr at room temperature followed by incubation with primary antibody at a 1:100 dilution (The monoclonal antibody against rat $sPLA_2$-IIA was a gift from Prof. Henk van den Bosch, Utrecht). The blot was incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G. After washing, peroxidase activity was detected by developing the blots according to the ECL method (Amersham-Pharmacia).

RESULTS

Effects of Phorbol Esters on the IL-1β-Induced mRNA Expression, Enzyme Secretion and Activity of $sPLA_2$IIA

As interleukin-1β is known to induce the expression and secretion of $sPLA_2$-IIA in mesangial cells starting 8–12 hr after treatment and reaching a plateau at 24 hr [14, 15], an incubation time of 24 hr was used in the following experiments. Incubation of cells with IL-1β (2 nM) stimulated the secretion of $sPLA_2$-IIA as shown by Western blot analysis (Fig. 1A, lane 2) and measurement of enzyme activity (Fig. 1B) in the cell culture supernatant. A 10-min pretreatment with different concentrations of phorbol ester

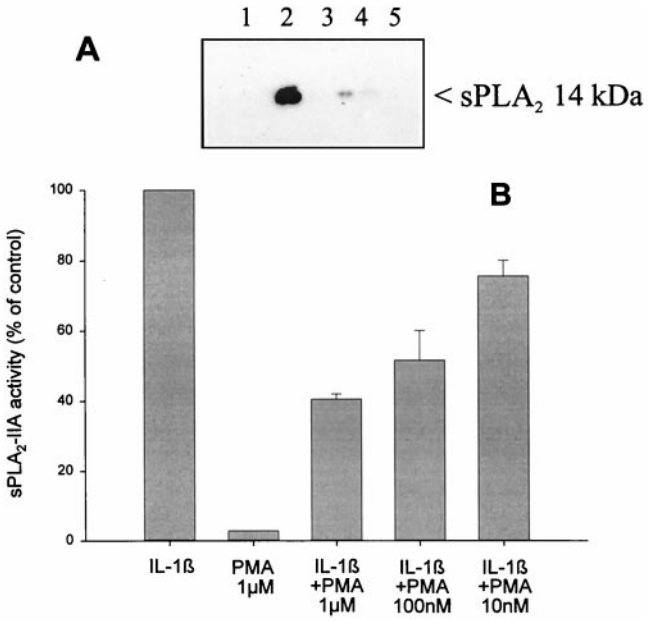

FIG. 1. Influence of a short-term preincubation with different PMA concentrations on IL-1β-induced $sPLA_2$-IIA secretion (A) and activity (B). (A) After incubation for 24 hr in Dulbecco's modified Eagle's medium containing 0.1 mg/mL BSA, mesangial cells were pretreated for 10 min with PMA (lanes 3 and 4—100 nM, lane 5—1 μM) or 0.01% dimethylsulphoxide as control (lanes 1 and 2). Then, cells were incubated for 24 hr with IL-1β (2 nM; lanes 2, 4, and 5) or vehicle (lane 1). Aliquots from the supernatants were taken for detection of $sPLA_2$-IIA protein by Western blotting. (B) Cells were pretreated for 10 min with the indicated PMA concentrations or 0.01% dimethylsulphoxide as control. They were then incubated for 24 hr with IL-1β (2 nM) or vehicle. Aliquots from the supernatants were taken to measure $sPLA_2$-IIA activity as described in Methods. Each value represents the means of 3 parallel determinations ± SE per experiment. The experiment was repeated 3 times with similar results.

PMA (Fig. 1) resulted in an approximate 90% reduction in the cytokine-stimulated secretion of $sPLA_2$-IIA at 100 nM PMA, which reached 100% at 1 μM (Fig. 1A, lanes 4 and 5). PMA alone did not stimulate $sPLA_2$-IIA secretion (Fig. 1A, lane 3). The $sPLA_2$-IIA activity (Fig. 1B) decreased dose dependently and was maximally reduced at 1 μM. The degree of inhibition by PMA varied from 60% to 80% in different experiments. The effects were weaker when PMA was added simultaneously with IL-1β and was more pronounced when given 10 min prior to the cytokine. PDBu, another biologically active phorbol ester, similarly inhibited cytokine-induced $sPLA_2$-IIA activity, whereas the inactive phorbol ester 4α-PDD had no effect (data not shown). All phorbol esters, when given alone, did not stimulate $sPLA_2$-IIA-activity.

In order to evaluate whether this inhibition of $sPLA_2$-IIA activity is due to regulation of $sPLA_2$-IIA gene expression, Northern blot analysis was performed. The data in Fig. 2 show that $sPLA_2$-IIA mRNA was present in IL-1β-stimulated cells as a single band of approx. 0.9 kb (Fig. 2, lane 2) when using a cDNA for rat $sPLA_2$-IIA [12]. In

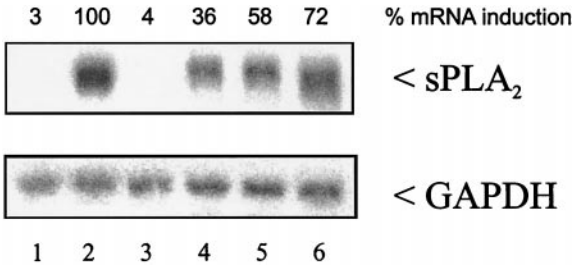

FIG. 2. Effect of different PMA concentrations on IL-1β-induced $sPLA_2$-IIA mRNA expression. Cells were preincubated with different concentrations of PMA or 0.01% dimethylsulfoxide as control for 10 min and then treated with IL-1β (2 nM) or vehicle for 24 hr. Lane 1—control; lane 2—IL-1β; lane 3—PMA (1 μM; lane 4—IL-1β/1 μM PMA; lane—IL-1β/100 nM PMA; lane 6—IL/1β/10 nM). Quantification of the filters was performed densitometrically with the BAS 1500 phosphorimager. To correct for differences in loading, the signal density of each RNA sample hybridized was divided by that hybridized to the GAPDH probe. The amount of mRNA calculated for $sPLA_2$-IIA in IL-1β-stimulated cells is expressed as 100%.

unstimulated cells there was no detectable $sPLA_2$-IIA mRNA (Fig. 2, lane 1). Incubation of cells with PMA dose dependently inhibited the accumulation of IL-1β-induced $sPLA_2$-IIA mRNA (Fig. 2, lanes 4–6). PMA alone did not induce $sPLA_2$-IIA mRNA (Fig. 2, lane 3). In summary, these results suggest that phorbol ester-activated PKC isoenzymes interfere with IL-1β-stimulated $sPLA_2$-IIA expression.

Effect of Down-regulation of PKC on IL-1β-Stimulated $sPLA_2$-IIA Induction

We have shown previously by Western blot analysis that mesangial cells express four PKC isoforms, PKC-α, -δ, -ε, and -ζ, which displayed distinctly different down-regulation kinetics on exposure to different concentrations of PMA [5–7]. An 8-hr treatment with 500 nM PMA was sufficient to completely deplete mesangial cells of PKC-α and -δ isotypes, and a 24-hr incubation with this PMA concentration was necessary to down-regulate PKC-ε [5]. In order to sequentially down-regulate the phorbol ester-sensitive PKC isoenzymes -α, -δ, and -ε, mesangial cells were pretreated for different periods (2–24 hr) with PMA. A 2- to 4-hr pretreatment with PMA (500 nM), which is known to down-regulate PKC-α completely [5], was not sufficient to eliminate the inhibitory effect on the subsequent IL-1β-stimulated $sPLA_2$-IIA mRNA expression (Fig. 3A, lanes 3 and 4) and activity (Fig. 3B). When cells were pretreated for 8 hr with PMA, which also down-regulates PKC-δ, $sPLA_2$-IIA mRNA induction recovered back to the level of IL-1β-stimulated induction (Fig. 3A, lane 5), whereas $sPLA_2$-IIA activity was still inhibited (Fig. 3B). However, when cells were pretreated for 24 hr with PMA which, in addition to PKC-α and -δ, causes an almost complete down-regulation of PKC-ε, an approx. 3-fold potentiation of $sPLA_2$-IIA mRNA levels was observed (Fig. 3A, lane 6), and $sPLA_2$-IIA activity nearly reached the levels obtained

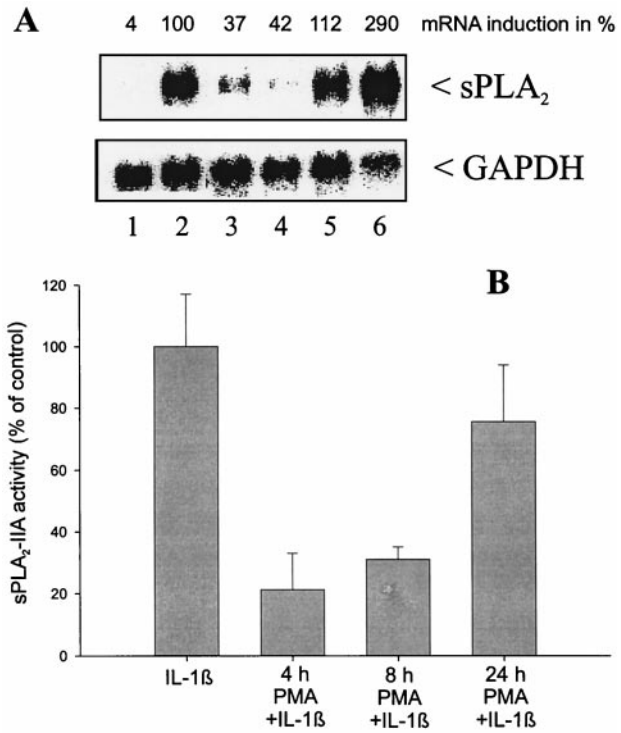

FIG. 3. Effect of down-regulation of PMA-sensitive protein kinase C isoenzymes at different preincubation times with PMA on IL-1β-induced $sPLA_2$-IIA mRNA induction (A) and activity (B). (A) Cells were preincubated for 2 hr (lane 3), 4 hr (lane 4), 8 hr (lane 5), or 24 hr (lane 6) with PMA (500 nM) and then treated for an additional 24 hr with IL-1β (2 nM, lanes 2–6) or vehicle as control (lane 1). Total cellular RNA was extracted and hybridized successively to ^{32}P-labelled $sPLA_2$-IIA and GAPDH cDNA probes as described in Methods. Quantification was performed as described in the legend of Fig. 2. The amount of mRNA calculated for $sPLA_2$-IIA in IL-1β-stimulated cells is expressed as 100%. (B) Cells were pretreated with 500 nM PMA for the indicated periods and IL-1β (2 nM) was added for an additional 24 hr. Aliquots of the supernatant were used to measure the $sPLA_2$-IIA activity as described in Methods. Each value represents the means of 3 parallel determinations ± SE per experiment. The experiment was repeated 3 times with similar results.

after IL-1β induction in non-PMA-treated cells (Fig. 3B). These results again indicate that PKC-ϵ in particular seems to be responsible for negative regulation of the $sPLA_2$-IIA expression seen in cytokine-stimulated mesangial cells.

IL-1β-Induced $sPLA_2$-IIA Activity and mRNA Expression Modulated by Thrombin

Thrombin is an important physiological agonist in the kidney and was shown to activate $cPLA_2$ and prostaglandin E_2 formation in mesangial cells [8]. We used thrombin as a physiological activator of PKC, as up to now the influence of thrombin on $sPLA_2$-IIA expression in mesangial cells has not been investigated. We found that thrombin, similarly to PMA, inhibited the IL-1β-induced increase in $sPLA_2$-IIA mRNA levels (Fig. 4A), enzyme secretion (Fig. 4B), as well as activity (Fig. 4C). Thrombin alone had no effect on

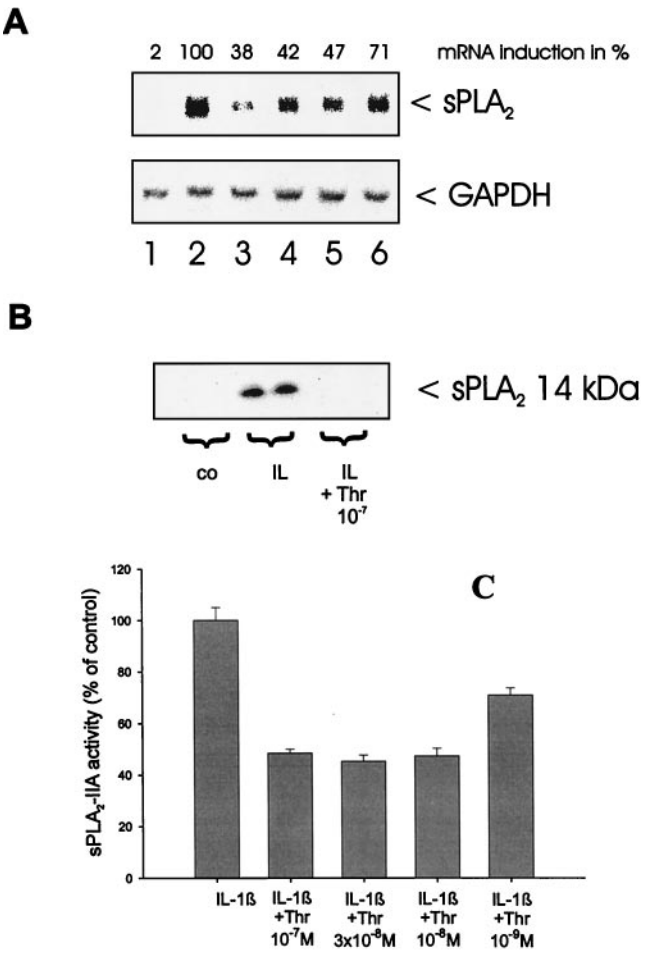

FIG. 4. Influence of thrombin on IL-1β-stimulated $sPLA_2$-IIA mRNA expression (A), enzyme secretion (B), and activity (C) in mesangial cells. Cells were pretreated for 15 min with different concentrations of thrombin and then incubated for 24 hr with IL-1β (2 nM). (A) Total cellular RNA was successively hybridized to ^{32}P-labelled $sPLA_2$-IIA and GAPDH cDNA probes as described in Methods. Quantification was performed as described in the legend of Fig. 2. The amount of mRNA calculated for $sPLA_2$-IIA in IL-1β-stimulated cells is expressed as 100%. Lane 1—vehicle; lane 2—IL-1β; lane 3—IL-1β/thrombin 10^{-7} M; lane 4—IL-1β/thrombin 3×10^{-8} M; lane 5—IL-1β/thrombin 10^{-8} M; lane 6—IL-1β/thrombin 10^{-9} M. (B) Aliquots from the supernatants were taken for detection of $sPLA_2$-IIA protein by Western blotting. In the graph double determinations were shown. (C) $sPLA_2$-IIA activity was measured in aliquots of cell culture supernatants as described in Methods. Each value represents the means of 3 parallel determinations ± SE per experiment. The experiment was repeated 3 times with similar results. Thr = thrombin; co = control.

$sPLA_2$-IIA expression (data not shown). The results suggest that thrombin may function as a physiological modulator of transcription of the proinflammatory $sPLA_2$-IIA.

Effect of Calphostin C on IL-1β- and Thrombin-Modulated $sPLA_2$-IIA Expression

Coincubation of mesangial cells with IL-1β and calphostin C resulted in a potentiation of $sPLA_2$-IIA mRNA expres-

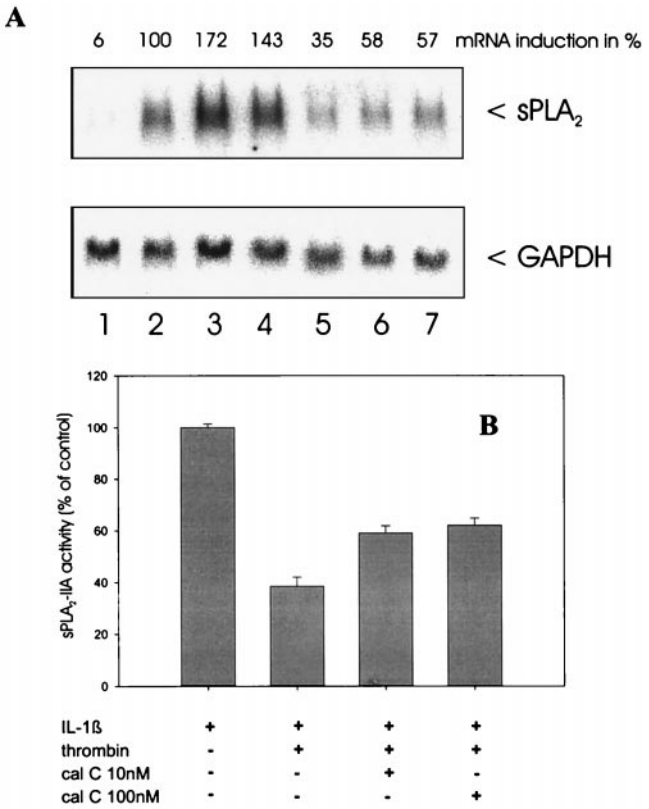

FIG. 5. Effect of calphostin C on the thrombin-induced inhibition of $sPLA_2$-IIA mRNA expression (A) and activity (B) stimulated by IL-1β. Cells were pretreated for 30 min with different calphostin C concentrations as indicated. Then, they were incubated for 24 hr with IL-1β (2 nM) in the absence or presence of thrombin (10^{-7} M). (A) Total cellular RNA was successively hybridized to ^{32}P-labelled $sPLA_2$-IIA and GAPDH cDNA probes as described in Methods. Quantification was performed as described in the legend of Fig. 2. The amount of mRNA calculated for $sPLA_2$-IIA in IL-1β-stimulated cells is expressed as 100%. Lane 1—vehicle; lane 2—IL-1β; lane 3—IL-1β/calphostin C 10 nM; lane 4—IL-1β/calphostin C 100 nM; lane 5—thrombin; lane 6—IL-1β/thrombin/calphostin C 10 nM; lane 7—IL- 1β/thrombin/calphostin C 100 nM. (B) $sPLA_2$-IIA activity was measured in aliquots of cell culture supernatants as described in Methods. Each value represents the means of 3 parallel determinations ± SE per experiment. The experiment was repeated 4 times with similar results. cal C = calphostin C.

sion by about 40 to 70% (Fig. 5A, lanes 3 and 4) as compared to IL-1β alone (lane 2). There was also a tendency for $sPLA_2$-IIA activity to increase, although not significantly (Table 1). *In vitro* incubation of $sPLA_2$-IIA with calphostin C had no effect on the enzyme activity (data not shown). When cells were treated with a combination of IL-1β, calphostin C, and thrombin, the inhibitory effect of thrombin on $sPLA_2$-IIA expression (Fig. 5A, lane 5) was partially reversed by the PKC inhibitor (Fig. 5A, lanes 6 and 7). Similar results were obtained by measuring $sPLA_2$-IIA activity (Fig. 5B). These data again indicate a negative modulatory role of PKC in IL-1β-stimulated $sPLA_2$-IIA expression.

TABLE 1. Influence of calphostin C on IL-1β-stimulated $sPLA_2$-IIA activity in mesangial cells

Treatment	$sPLA_2$-IIA activity (image quants)
control	249 ± 26
IL-1β	2191 ± 16
IL-1β + calphostin C (10 nM)	2732 ± 313 (NS)
IL-1β + calphostin C (100 nM)	2765 ± 136 (NS)

Cells were pretreated for 30 min with different calphostin C concentrations as indicated and were then incubated for 24 hr with IL-1β (2 nM). $sPLA_2$-IIA activity was measured in aliquots of cell culture supernatants as described in Methods. Each value represents the means of 3 parallel determinations ± SE per experiment. The experiment was repeated 3 times with similar results. Statistical analysis of values obtained from calphostin C-treated cells compared to IL-1β was performed by Student's *t*-test. NS = not significant.

DISCUSSION

In this study, we report for the first time that activation of PKC by either phorbol esters or the physiological agonist thrombin negatively regulates gene expression of $sPLA_2$-IIA in cytokine-stimulated mesangial cells, leading to a reduced secretion of enzyme and diminished activity in the culture supernatant. Mesangial cells express the phorbol ester-sensitive PKC-α, -δ, and -ε isoenzymes and the phorbol ester-insensitive isoenzyme PKC-ζ. Only the former could be investigated by using PMA or PDBu as activators. These isoenzymes are differentially regulated by phorbol esters and display distinctly different depletion kinetics, as was shown by our group by performing Western blot analysis using specific antibodies against the different PKC isoenzymes [5–7]. It was shown that different periods of PMA treatment of mesangial cells resulted in a rapid activation of PKCs followed by sequential down-regulation of the different isoenzymes. This observation was useful to study the effects of distinct PKC isoenzymes on the IL-1β-induced activation of $sPLA_2$-IIA. We found that $sPLA_2$-IIA mRNA expression and activity were reduced by PMA treatment of mesangial cells for periods of 10 min to 4 hr, time periods required to activate and subsequently down-regulate PKC-α and -δ. Inhibition of $sPLA_2$-IIA activity started to recover after 8 hr of phorbol ester treatment, reaching a 3-fold potentiation after a 24-hr period of PMA pretreatment, i.e. at a time point where PKC-ε was also down-regulated. These data suggest that PKC-ε plays a role as a negative regulator of IL-1β-induced $sPLA_2$-IIA expression in mesangial cells in a way similar to that reported for other proinflammatory mediators such as the inducible isoform of nitric oxide synthase [16]. The marked potentiation of IL-1β-induced $sPLA_2$-IIA mRNA levels in PKC-depleted cells contrasts with the moderate up-regulation of $sPLA_2$-IIA activity in the cell culture supernatants (Figs. 2 and 4). This points to possible additional effects of PKC isoenzymes on $sPLA_2$-IIA synthesis and/or secretion. Further experiments are in progress to evaluate such effects.

The mode of cross-communication between PKC in the IL-1β signal transduction pathway is still unclear. One

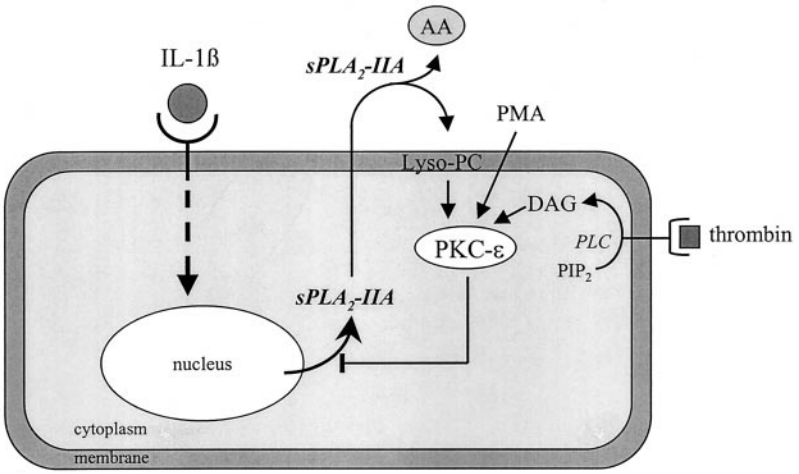

FIG. 6. Interaction between IL-1β-stimulated sPLA$_2$-IIA induction and PKC-ε activated by PMA or thrombin. AA, arachidonic acid; DAG, diacylglycerol; lyso-PC, lysophosphatidylcholine; PIP$_2$, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C.

possibility is that the regulation occurs on the transcriptional level, and therefore PKC was proposed to interfere with mechanisms leading to activation of transcription factors. Studies by Walker *et al.* [17] have shown that NFκB activation is an essential component of the cytokine signalling pathway responsible for sPLA$_2$-IIA gene regulation. The phosphorylation of IκB (inhibitor of NFκB) was identified as one step in the activation process, and it was discussed earlier whether PKC may be indirectly responsible for this phosphorylation and subsequent liberation of active NFκB [18]. However, it is known that depletion of PKC by chronic PMA treatment or by PKC inhibitors did not affect NFκB activation by IL-1β or tumor necrosis factor-α, indicating that it is independent of the action of PMA-sensitive PKC isoenzymes [19, 20]. Recently, novel kinases unrelated to PKC have been identified as being responsible for phosphorylation of IκB [21]. In addition to NFκB, the sPLA$_2$-IIA promoter has a potential binding site for the transcription factors AP-1 and -2. PKC is known to activate transcription factor AP-1 [22], although a regulatory function of AP-1 or -2 on sPLA$_2$-IIA gene expression is presently unknown.

A further possibility as to where PKC might interfere upstream at the very beginning of the signalling cascade is the activation of IL-1RI. IL-1β signaling occurs exclusively via the type I receptor (for review see [23]). Horuk and Gross [24] reported that phorbol ester treatment reduced IL-1β binding by down-regulation of IL-1RI. This effect could be reversed by prior incubation with a specific PKC inhibitor. The mechanism by which PKC reduces IL-1β binding and receptor inactivation is unknown. Phosphorylation of IL-1RI was reported after exposure to IL-1β [25] and amino acid sequence comparisons of the cytosolic domain of the IL-1RI have shown similarities with a PKC acceptor site. However, the significance of this observation is unclear. Studies by Kuno *et al.* [26] have shown that PKC acceptor sites were not required for the transducing activity of IL-1RI. IL-1β was shown to increase IL-1RI mRNA levels in mesangial cells; however, platelet-derived growth factor, a potent activator of PKC-α, -δ, and ε, had no effect on IL-1RI mRNA expression [27], thus making it less likely that PKC interferes at the level of IL-1RI in mesangial cells.

The amplification of signals triggered by IL-1β and leading to NFκB activation requires activity of IRAKs [28] as well as the IL-1RI accessory protein [29, 30]. An attractive hypothesis is that these molecules may serve as direct or indirect targets for PKC. Multiple phosphorylations of IRAKs take place after IL-1RI binding before they are degraded by the proteasome complex [31]. A comparison of IL-1β-induced phosphorylations with those induced by phorbol esters via PKC shows some similarities. The functional consequence, i.e. activation or inactivation of IRAKs or one of the downstream molecules/kinases by PKC-mediated phosphorylation, is completely unknown. Recently, evidence for a physical and functional interaction of the IL-1RI with phosphatidylinositol 3-kinase was reported, suggesting that this enzyme seems to cooperate with other IL-1β-induced signals to activate AP-1 as well as NFκB [32]. Therefore, phosphatidylinositol 3-kinase is another interesting target of phorbol ester-activated PKCs that may interfere with IL-1RI signalling, leading to sPLA$_2$-IIA expression.

A physiological activator of PKC in different cell systems, including mesangial cells, is thrombin. The role of thrombin in glomerular dysfunction is not well defined. However, the effects of the actions of thrombin such as intraglomerular thrombosis and fibrin deposition are common characteristics of glomerular injury (for review see [33]). Thrombin is known to act as a mitogen in mesangial cells. Furthermore, it stimulates the production of transforming growth factor-β, increases intracellular Ca^{2+} levels, and activates cPLA$_2$ and prostaglandin biosynthesis [8, 34, 35]. The signalling of thrombin after activation of its receptor in mesangial cells seems to be mediated through activation of PKC [36].

This activation might be important during inflamma-

tion, tissue remodelling, and wound repair. In this study, we found that thrombin, as does PMA, inhibits $sPLA_2$-IIA induction very potently, probably via activation of PKC. To corroborate these findings we used the PKC inhibitor calphostin C [37]. This compound was reported to inhibit vesicle-mediated secretory processes [38]; however, we did not find any inhibitory effect of calphostin C on $sPLA_2$-IIA secretion, as was shown by the activity measurements in the supernatants under this condition (Table 1). On the contrary, with calphostin C we observed a partial reconstitution of IL-1β-induced $sPLA_2$-IIA induction in mesangial cells exposed to thrombin. These data suggest that the thrombin effect is indeed partially mediated by activation of calphostin C-sensitive PKC isoenzymes but that additional components also contribute to its inhibitory action. Thrombin is known to stimulate the secretion of platelet-derived growth factor [39], which in turn may inhibit $sPLA_2$-IIA induction [40]. The inhibition of IL-1β-induced $sPLA_2$-IIA secretion by thrombin may represent an attenuating mechanism for acute inflammatory processes. In mesangial cells, extracellular secretory phospholipases A_2, in addition to their lipolytic activity, also have the capacity to act as a mitogen in stimulating the c-Raf-1/mitogen-activated protein kinase cascade [4] and inducing their own expression [41], suggesting a role for the propagation of inflammation.

Therefore, thrombin, via activation of PKC, may act in line with other potent physiological inhibitors of IL-1β-induced $sPLA_2$-IIA synthesis such as transforming growth factor-β_2 [42] and platelet-derived growth factor [40] to protect the kidney from damage resulting from cytokine-stimulated $sPLA_2$-IIA release and subsequent inflammatory reactions. In particular, transforming growth factor-β production in mesangial cells is known to be stimulated by thrombin via PKC-dependent mechanisms [35]. The scheme in Fig. 6 illustrates the interaction between IL-1β-stimulated $sPLA_2$-IIA induction and PKC-ε activated by PMA or thrombin. Further detailed examinations are currently underway in our laboratory to evaluate the mechanism of PKC-mediated modulation of IL-1β signal transduction in mesangial cells.

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