Cyclic AMP mimics, but does not mediate, interleukin-1- and tumour-necrosis-factor-stimulated phospholipase A₂ secretion from rat renal mesangial cells

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We have previously shown that recombinant interleukin 1 (IL-1) and recombinant tumour necrosis factor (TNF) synergistically stimulate phospholipase A₂ release from mesangial cells. We now report that treatment of mesangial cells with the β -agonist salbutamol, prostaglandin E₂ (PGE₂), cholera toxin or forskolin, which all activate adenylate cyclase, increased release of phospholipase A₂ activity. Likewise, addition of a membrane-permeant cyclic AMP (cAMP) analogue or the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine enhanced release of phospholipase A₂ activity from mesangial cells. There was a lag period of about 8 h before a significantly enhanced secretion could be detected. Furthermore, actinomycin D or cycloheximide completely suppressed cAMP-stimulated secretion of phospholipase A₂. Angiotensin II, the phorbol ester phorbol 12-myristate 13-acetate, the Ca²⁺ ionophore A23187 and a membrane-permeant cGMP analogue did not stimulate phospholipase A₂ release from the cells. Treatment with indomethacin completely inhibited IL-1 β - and TNF-stimulated PGE₂ synthesis, without having any effect on phospholipase A₂ secretion, thus excluding cytokine-induced PGE₂ synthesis as the mediator of phospholipase A₂ release. Neither IL-1 β nor TNF induced any increase in intracellular cAMP in mesangial cells. Furthermore, incubation of the cells with 2',5'-dideoxyadenosine, an inhibitor of adenylate cyclase, did not block cytokine-stimulated phospholipase A₂ secretion. In addition, IL-1 β and TNF synergistically interacted with forskolin to stimulate phospholipase A₂ release from the cells. The protein kinase inhibitors H-8, staurosporine, K252a and amiloride inhibited IL-1 β - and TNF-stimulated phospholipase A₂ secretion. However, high concentrations that inhibit other protein kinases were needed. These observations suggest that IL-1 β and TNF cause secretion of phospholipase A₂ by a mechanism independent of cAMP. The signalling pathways used by IL-1 β and TNF may involve a protein kinase that is probably different from protein kinase A or protein kinase C.

INTRODUCTION

IL-1 and TNF are polypeptide mediators produced by a variety of cell types, and are a central factor in the development of immune and inflammatory processes (see [1–4] for reviews). Other reports have demonstrated that mesangial cells also express and secrete IL-1 [5,6] and TNF [7]. The IL-1 secreted by the cells was shown to stimulate proliferation in an autocrine manner [8,9]. Thus the local release of growth factors such as IL-1 may contribute to the development of glomerular hypercellularity, a characteristic feature of many forms of immune-mediated glomerulonephritis [10]. Werber *et al.* [6] reported augmented IL-1 gene expression in glomerular mesangial cells in immune-complex glomerulonephritis. Furthermore, IL-1 stimulates the release of a specific type IV collagenase [11], and both cytokines, IL-1 and TNF, enhance the synthesis of prostaglandins by mesangial cells [12–14]. The interaction of monocytes, which represent the dominant cell type infiltrating the glomerulus in immune-mediated glomerulonephritis, and intrinsic mesangial cells plays a central role in the pathogenesis of these diseases. Recently, we have shown that a soluble phospholipase A₂ is secreted from mesangial cells in response to IL-1 α , IL-1 β and TNF [13]. Extracellular phospholipase A₂ is proinflammatory and vasoactive, and mediates hyperaemia and a marked inflammatory reaction when injected intracutaneously into rabbits [15] or intra-articularly into rats [16]. In the present work we tried to gain further insight into the signalling mechanism involved in

IL-1 β - and TNF-stimulated phospholipase A₂ release from mesangial cells.

MATERIALS AND METHODS

Cell culture

Rat mesangial cells were cultivated as described previously [17,18]. The cells were grown in RPMI 1640 supplemented with 20% (v/v) fetal-calf serum, penicillin (100 units/ml), streptomycin (100 μ g/ml) and bovine insulin at 0.66 unit/ml (Sigma). Mesangial cells were characterized morphologically by phase-contrast microscopy, positive staining for the intermediate filaments desmin and vimentin, which is considered to be specific for myogenic cells [19], and negative staining for Factor VIII-related antigen and cytokeratin, excluding endothelial and epithelial contamination respectively. For the experiments passages 6–32 of mesangial cells were used.

Incubation of cells and prostaglandin analysis

Confluent mesangial cells cultured in 16 mm-diameter wells were washed twice with phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/8 mM-Na₂HPO₄/1.5 mM-KH₂PO₄) and incubated with 1 ml of Dulbecco's modified Eagle medium containing 0.1 mg of fatty-acid-free BSA (Sigma)/ml with or without the different agents. After the indicated time periods, the medium was withdrawn and centrifuged in an IEC Centra-7R laboratory

Abbreviations used: IL-1, interleukin 1; TNF, tumour necrosis factor; cAMP, cyclic AMP; cGMP, cyclic GMP; Bt₂cAMP, N⁶,2'-O-dibutyryl cAMP; Bt₂cGMP, N⁶,2'-O-dibutyryl cGMP; H-8, N-[(2-methylamino)ethyl]-5-isoquinolinesulphonamide dihydrochloride; PMA, 12-myristate 13-acetate; PG, prostaglandin.

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centrifuge. The supernatant was removed and frozen in liquid nitrogen and stored at -80°C until assay of phospholipase A_2 and PGE_2 . PGE_2 was determined by radioimmunoassay (New England Nuclear). Cells were dissolved in 0.5 M-NaOH and protein was determined by the Lowry method [20], with BSA as a standard.

Phospholipase A_2 assay

Phospholipase A_2 activity was determined with $[1\text{-}^{14}\text{C}]\text{oleate}$ -labelled *Escherichia coli* as substrate as described previously [21]. The substrate was prepared by growing *E. coli* in the presence of $[1\text{-}^{14}\text{C}]\text{oleate}$, followed by autoclaving to inactivate endogenous phospholipases. Over 95% of the label incorporated by *E. coli* was in phospholipid and, as demonstrated by hydrolysis with snake venom (*Crotalus adamanteus*), more than 95% of the $[1\text{-}^{14}\text{C}]\text{oleate}$ was in the *sn*-2 position of the phospholipids [21]. Assay mixtures (1.0 ml) contained 100 mM-Tris/HCl buffer (pH 7.0), 1.0 mM- CaCl_2 , 2.5×10^8 $[1\text{-}^{14}\text{C}]\text{oleate}$ -labelled *E. coli* (5 nmol of phospholipid, 5000–8000 c.p.m.) and the enzyme to be tested at a dilution producing 10–15% substrate hydrolysis. Reaction mixtures were incubated for 1 h at 37°C in a shaking water bath. The reaction was stopped by the addition of 5 ml of propan-2-ol/*n*-heptane/1 M- H_2SO_4 (40:10:1, by vol.) followed by 2 ml of heptane and 3 ml of water. After vigorous shaking and phase separation, a portion (2.5 ml) of the upper phase was passed over a column of silicic acid (3.5 cm $\times$ 0.5 cm). Free $[1\text{-}^{14}\text{C}]\text{oleic}$ acid was quantitatively eluted with 1 ml of ethyl acetate. Radioactivity was determined in a scintillation counter. Phospholipase A_2 activity is expressed as $[1\text{-}^{14}\text{C}]\text{oleate}$ radioactivity released by 100 μl of cell culture supernatant.

cAMP determination

Confluent mesangial cells in 35 mm-diameter dishes were incubated with IL-1 β or TNF for the indicated time periods. Reactions were terminated by aspiration of the medium and addition of 1 ml of ice-cold 5% (w/v) trichloroacetic acid. For extraction of cAMP, the dishes were put on ice for 1 h. The trichloroacetic acid was then removed by extraction with 4 $\times$ 5 vol. of water-saturated diethyl ether, and cAMP was determined by radioimmunoassay (New England Nuclear).

Statistics

Statistical analysis was by Student's *t* test, and $P < 0.05$ was used as the criterion for statistical significance.

Chemicals

$[1\text{-}^{14}\text{C}]\text{Oleic}$ acid was purchased from Amersham International, Amersham, Bucks., U.K.; PMA, forskolin and 1,9-dideoxy-forskolin were from Calbiochem, Lucerne, Switzerland; recombinant human IL-1 β , indomethacin, salbutamol and staurosporine were prepared by Ciba-Geigy Ltd., Basel, Switzerland; recombinant human TNF was from Boehringer, Mannheim, Germany; 2',5'-dideoxyadenosine was from Pharmacia Dübendorf, Switzerland; amiloride, PGE_2 , A23187, 3-isobutyl-1-methylxanthine, cholera toxin, actinomycin D, cycloheximide, Bt_2cAMP , Bt_2cGMP and angiotensin II were from Sigma, St. Louis, MO, U.S.A.; *N*-[2-(methylamino)ethyl]-5-isoquinolinesulphonamide dihydrochloride (H-8) was from Seikagaku Kogyo Co., Tokyo, Japan.

RESULTS

cAMP stimulates phospholipase A_2 release from mesangial cells

In view of the stimulatory effect of IL-1 and TNF on phospholipase A_2 release from mesangial cells [13], we started a series of experiments to investigate the possible signalling mech-

Fig. 1. Time course of basal and forskolin-stimulated phospholipase A_2 release from mesangial cells

Confluent mesangial cells were incubated with forskolin (10 μM) (●) or vehicle (○) for the indicated time periods. Thereafter phospholipase A_2 activity was determined in the medium as outlined in the Materials and methods section. Results are means from four experiments; S.E.M. ranged between 2% and 19% of the radioactivity at each point.

anism involved in this cell response. As shown in Fig. 1 and Table 1 mesangial cells spontaneously released phospholipase A_2 activity at a low rate. Addition of forskolin (10 μM), a potent activator of adenylate cyclase, resulted in a significant increase in the rate of phospholipase A_2 secretion after a lag time of 8 h. A similar lag period of 8 h has been observed for IL-1 β -stimulated phospholipase A_2 release [13]. The response to forskolin was dose-dependent, with a threshold at 0.1 μM -forskolin, and maximal effect at 100 μM -forskolin. Like the IL-1 β -induced phospholipase A_2 [13], the forskolin-induced enzyme had a neutral pH optimum and the activity was critically dependent on free

Table 1. Effect of different compounds on phospholipase A_2 release from mesangial cells

Confluent mesangial cells were incubated with the indicated compounds for 24 h. After the incubation period, the medium was removed and phospholipase A_2 activity was determined as outlined in the Materials and methods section. Results are means $\pm$ S.E.M. ($n = 4$).

Addition	$[^{14}\text{C}]\text{Oleate}$ radioactivity (c.p.m.)
Control	558 $\pm$ 17
Forskolin (10 μM)	1376 $\pm$ 21
1,9-Dideoxyforskolin (10 μM)	585 $\pm$ 65
Cholera toxin (1 $\mu\text{g}/\text{ml}$)	1457 $\pm$ 22
Salbutamol (1 μM)	1480 $\pm$ 31
Bt_2cAMP (1 mM)	1425 $\pm$ 24
3-Isobutyl-1-methylxanthine (1 mM)	905 $\pm$ 29
Actinomycin D (10 μM)	224 $\pm$ 13
Cycloheximide (10 μM)	219 $\pm$ 21
Forskolin (10 μM) + actinomycin D (10 μM)	255 $\pm$ 17
Forskolin (10 μM) + cycloheximide (10 μM)	281 $\pm$ 16
Angiotensin II (100 nM)	621 $\pm$ 27
PMA (100 nM)	536 $\pm$ 25
A23187 (10 μM)	585 $\pm$ 11
PMA (10 nM) + A23187 (1 μM)	528 $\pm$ 18
Bt_2cGMP (1 mM)	631 $\pm$ 46
PGE_2 (1 μM)	1758 $\pm$ 77

Ca²⁺ concentration, with significant activity in the low-micromolar range (results not shown). The 1,9-dideoxy derivative of forskolin, which is unable to activate adenylate cyclase [22], had no effect on phospholipase A₂ release from mesangial cells (Table 1), thus indicating that it was not a non-specific effect of forskolin that caused the enhanced secretion of phospholipase A₂ from mesangial cells. The diterpene forskolin is known to activate adenylate cyclase by direct stimulation of the catalytic subunit of the enzyme [22]. In a further approach we used salbutamol and cholera toxin, two compounds that activate the adenylate cyclase system by different mechanisms. Salbutamol binds to β -adrenergic receptors on the cell surface of mesangial cells and activates adenylate cyclase via the stimulatory G-protein, G_s [22]. Cholera toxin causes ADP-ribosylation of G_s, thus altering this G-protein to a state of permanent activation, leading to the subsequent stimulation of the catalytic moiety of adenylate cyclase. As shown in Table 1, both agents enhanced phospholipase A₂ release from mesangial cells. In a next step we used the membrane-permeant analogue of cAMP, Bt₂cAMP, to elevate the intracellular concentration of cAMP directly. The data in Table 1 clearly show that Bt₂cAMP increased the release of phospholipase A₂ activity. In addition, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, which increases cAMP by inhibiting the enzyme that degrades it, also enhanced phospholipase A₂ release (Table 1). As reported for the IL-1- and TNF-stimulated phospholipase A₂ release [13], addition of actinomycin D (10 μ M) or cycloheximide (10 μ M) at the time of stimulation completely suppressed the forskolin-induced enzyme release from mesangial cells (Table 1). Actinomycin D and cycloheximide also suppressed the basal rate of secretion of phospholipase A₂ (Table 1).

Stimulation of phosphoinositide hydrolysis, protein kinase C, Ca²⁺ mobilization and cGMP generation has no effect on phospholipase A₂ release

It has been reported that the chemotactic peptide fMet-Leu-Phe stimulates phospholipase A₂ secretion from rabbit peritoneal neutrophils [23]. fMet-Leu-Phe is a potent activator of phospholipase C-mediated phosphoinositide hydrolysis in neutrophils [24,25]. In order to elucidate a possible involvement of phosphoinositide turnover in phospholipase A₂ secretion from mesangial cells, we tested the effect of angiotensin II, which is known to activate phospholipase C in these cells (see [26] for review). Furthermore, we checked the effects of PMA, a potent activator of protein kinase C, and the Ca²⁺ ionophore A23187 on enzyme secretion. As shown in Table 1, neither angiotensin II (100 nM) or PMA (100 nM) or A23187 (10 μ M), nor a combination of PMA and A23187, had any effect on phospholipase A₂ release from mesangial cells, thus excluding a possible contribution of phospholipase C-triggered signalling pathways. In addition, the membrane-permeant analogue of cGMP, Bt₂cGMP, was equally ineffective in stimulating phospholipase A₂ release (Table 1). From all these results it is tempting to speculate that cAMP may be the messenger of IL-1- and TNF-stimulated phospholipase A₂ secretion from mesangial cells.

Inhibition of prostaglandin synthesis does not inhibit phospholipase A₂ release

Previously we have shown that IL-1 and TNF synergistically stimulate release of phospholipase A₂ as well as PGE₂ from the mesangial cells [13]. As PGE₂ and PGI₂ have been shown to stimulate cAMP generation in mesangial cells [27], one might assume that cytokine-stimulated PG formation and subsequent cAMP generation is responsible for the observed phospholipase A₂ secretion. However, PG concentrations as high as 1 μ M, two to three orders of magnitude higher than the values found after

Fig. 2. Effect of indomethacin on IL-1 β - and TNF-stimulated phospholipase A₂ release and PGE₂ synthesis in mesangial cells

Confluent mesangial cells were incubated for 24 h with IL-1 β (1 nM) (○, □) or TNF (1 nM) (●, ■) with the indicated concentration of indomethacin. Thereafter the medium was withdrawn and phospholipase A₂ activity (□, ■) and PGE₂ concentrations (○, ●) were determined as outlined in the Materials and methods section. Results are means $\pm$ S.E.M. ($n = 4$).

cytokine stimulation, were needed in order to see significant increases in intracellular cAMP levels [27]. Table 1 shows that PGE₂ (1 μ M) stimulated phospholipase A₂ release from mesangial cells. The potent cyclo-oxygenase inhibitor indomethacin completely inhibited IL-1- and TNF-stimulated PGE₂ synthesis without having any significant effect on the cytokine-induced phospholipase A₂ secretion (Fig. 2). Similar results have been reported for the more potent cyclo-oxygenase inhibitor diclofenac [28]. This clearly excludes prostaglandins as mediators of IL-1- and TNF-induced phospholipase A₂ secretion from mesangial cells.

IL-1 and TNF do not increase cAMP in mesangial cells

To investigate more directly the possibility that IL-1 and TNF exert their stimulatory activity on phospholipase A₂ release by means of cAMP, we determined cAMP levels in mesangial cells stimulated with or without IL-1 β or TNF. Neither IL-1 β (1 nM)

Table 2. Effect of 2',5'-dideoxyadenosine on IL-1 β , TNF- and PGE₂-stimulated phospholipase A₂ release from mesangial cells

Confluent mesangial cells were incubated with the indicated compounds for 24 h. Thereafter the medium was removed and phospholipase A₂ activity was determined as outlined in the Materials and methods section. Results are means $\pm$ S.E.M. ($n = 4$).

Addition	[¹⁴ C]Oleate radioactivity (c.p.m.)
Control	678 $\pm$ 45
IL-1 β (0.1 nM)	3555 $\pm$ 153
TNF (1 nM)	4024 $\pm$ 288
PGE ₂ (1 μ M)	1828 $\pm$ 54
2',5'-Dideoxyadenosine (10 μ M)	715 $\pm$ 6
IL-1 β (0.1 nM) + 2',5'-dideoxyadenosine (10 μ M)	4975 $\pm$ 116
TNF (1 nM) + 2',5'-dideoxyadenosine (10 μ M)	5124 $\pm$ 214
PGE ₂ (1 μ M) + 2',5'-dideoxyadenosine (10 μ M)	755 $\pm$ 47

nor TNF (1 nM) had any significant effect on cAMP concentration in mesangial cells after incubation periods ranging from 1 min to 24 h (results not shown). Even the inhibition of the cAMP-degrading phosphodiesterase by 3-isobutyl-1-methylxanthine did not result in a significant cytokine-stimulated cAMP accumulation (results not shown). In contrast, forskolin (10 μ M) caused a 4-fold and 12-fold increase in cAMP in the absence or presence of 3-isobutyl-1-methylxanthine respectively (results not shown). It has been reported that cAMP-dependent protein kinases can be activated in stimulated cells without concomitant changes in cAMP levels, and it was concluded that dynamic cAMP turnover could be a means of signal amplification [29,30]. In order to eliminate convincingly cAMP as the mediator of IL-1 β - and TNF-mediated phospholipase A₂ secretion, we performed additional experiments. Further evidence against a role for cAMP as the messenger of IL-1 β - and TNF-stimulated phospholipase A₂ release comes from experiments with the adenylate cyclase inhibitor 2',5'-dideoxyadenosine [31–33], which has been shown to block PG-stimulated cAMP accumulation in mesangial cells [34]. Treatment of mesangial cells with 10 μ M-2',5'-dideoxyadenosine did not inhibit and, in fact, enhanced both IL-1 β - and TNF-induced phospholipase A₂ secretion, as shown in Table 2. In contrast, 2',5'-dideoxyadenosine not only blocked PGE₂-stimulated cAMP formation (results not shown), but also inhibited PGE₂-induced phospholipase A₂ secretion (Table 2).

Forskolin and IL-1 β or TNF synergistically interact to stimulate phospholipase A₂ secretion

Agonists that share identical signalling mechanisms should display additive effects when combined at sub-optimal con-

Fig. 3. Synergistic stimulation of phospholipase A₂ secretion from mesangial cells by IL-1 β and forskolin

Confluent mesangial cells were incubated for 24 h with the indicated concentrations of forskolin without (○) or with IL-1 β : ●, 10 pM; △, 100 pM; ▲, 500 pM; □, 2 nM; ■, 10 nM. After the incubation period, the medium was withdrawn and phospholipase A₂ activity was determined as outlined in the Materials and methods section. Results are means $\pm$ S.E.M. ($n = 4$).

Table 3. Synergistic effect of TNF and forskolin on phospholipase A₂ release from mesangial cells

Confluent mesangial cells were incubated with TNF (10 nM) or forskolin (100 μ M), separately or together, for 24 h. Thereafter the medium was removed and phospholipase A₂ activity was determined as outlined in the Materials and methods section. Results are means $\pm$ S.E.M. ($n = 4$).

Addition	[¹⁴ C]Oleate radioactivity (c.p.m.)
Control	490 $\pm$ 23
TNF	2139 $\pm$ 110
Forskolin	3350 $\pm$ 406
TNF + forskolin	36400 $\pm$ 650

centrations, but not at optimal doses. Fig. 3 shows that forskolin dose-dependently stimulated phospholipase A₂ secretion, reaching a plateau between 10 and 100 μ M. IL-1 β also stimulates phospholipase A₂ release in a dose-dependent manner. When IL-1 β and forskolin were added simultaneously, there was a synergistic interaction over the whole dose-response range, as shown in Fig. 3. Similarly, TNF and forskolin interact in a synergistic fashion to stimulate phospholipase A₂ release from mesangial cells (Table 3). These data provide further evidence that IL-1 and TNF use signalling pathways different from the adenylate cyclase system in order to stimulate phospholipase A₂ secretion from mesangial cells.

Protein kinase inhibitors attenuate phospholipase A₂ release

In a further approach we used different protein kinase inhibitors in an attempt to determine the signalling cascades in cytokine-stimulated phospholipase A₂ release. The isoquinoline H-8 has been reported to selectively inhibit cAMP-dependent and cGMP-dependent protein kinases, with K_i values of 1.2 μ M and 0.48 μ M respectively [35]. Fig. 4 shows that H-8 dose-dependently inhibited IL-1 β - and TNF-stimulated phospholipase A₂ release. However, concentrations as high as 50 μ M were needed to obtain a significant inhibition of phospholipase A₂ release. In addition, H-8 also blocked forskolin-stimulated phospholipase A₂ secretion, but again 50 μ M-H-8 was needed to

Fig. 4. Effect of H-8 on IL-1 β - and TNF-stimulated phospholipase A₂ release from mesangial cells

Confluent mesangial cells were incubated for 24 h with IL-1 β (500 pM) (●) or TNF (500 pM) (○) and the indicated concentrations of H-8. Thereafter the medium was removed and phospholipase A₂ activity was determined as outlined in the Materials and methods section. Results are means $\pm$ S.E.M. ($n = 4$).

Table 4. Effects of protein kinase inhibitors on IL-1 β -stimulated phospholipase A₂ release from mesangial cells

Confluent mesangial cells were incubated with the indicated compounds for 24 h. After the incubation period, the medium was removed and phospholipase A₂ activity was determined as outlined in the Materials and methods section. Results are means $\pm$ S.E.M. ($n = 4$).

Addition	[¹⁴ C]Oleate radioactivity (c.p.m.)
Control	481 $\pm$ 52
IL-1 β (0.1 nM)	1697 $\pm$ 55
Staurosporine (1 μ M)	241 $\pm$ 20
K-252a (1 μ M)	385 $\pm$ 55
Amiloride (50 μ M)	451 $\pm$ 45
IL-1 β (0.1 nM) + staurosporine (1 μ M)	364 $\pm$ 61
IL-1 β (0.1 nM) + K-252a (1 μ M)	441 $\pm$ 26
IL-1 β (0.1 nM) + amiloride (50 μ M)	1184 $\pm$ 38

block the response (results not shown). Therefore, these data do not necessarily argue against cAMP-dependent protein kinase mediating cytokine-induced enzyme release. Staurosporine and its derivative K-252a were reported as potent inhibitors of protein kinase C, with 50% inhibition at 2.7 nM and 25 nM respectively [36,37]. Both compounds also inhibited IL-1 β - and TNF-stimulated phospholipase A₂ release, as shown in Table 4. However, concentrations far beyond those needed to inhibit protein kinase C were necessary to inhibit cytokine-induced enzyme release. These results agree well with the lack of activity of PMA in inducing phospholipase A₂ secretion (Table 1). Together, these data suggest that protein kinase C is not involved in IL-1 β - and TNF-induced enzyme release. We next used the diuretic agent amiloride, a non-specific inhibitor of protein kinases, previously shown to block epidermal-growth-factor receptor autophosphorylation and serine/threonine phosphorylation in intact cells [38,39]. As shown in Table 4, amiloride attenuates IL-1 β - and TNF-induced phospholipase A₂ release.

DISCUSSION

Phospholipase A₂ is a lipolytic enzyme that cleaves the 2-acyl fatty acid ester of glycerophospholipids with subsequent liberation of free fatty acids, especially arachidonic acid for further metabolism to prostaglandins and other eicosanoids. Eicosanoids, as well as phospholipase A₂ itself, play an important role in inflammatory processes (see [40] for review). The extracellular secretion of a soluble phospholipase A₂ was first reported from stimulated macrophages [41] and neutrophils [23], and from aggregated platelets [42]. Stimulation and release of phospholipase A₂ by IL-1 and TNF was observed in rat and rabbit chondrocytes [43,44], as well as in human synovial cells [45,46]. Recently, we have reported that IL-1 and TNF interact synergistically to stimulate phospholipase A₂ secretion from rat glomerular mesangial cells [13]. This enzyme secretion may play a role in the propagation of inflammatory reactions within the glomerulus. The signal transduction mechanism by which IL-1 and TNF exert their effect on target cells is still largely unknown. In order to unveil the biochemical mechanisms whereby IL-1 and TNF cause phospholipase A₂ release from mesangial cells, we tested several compounds known to activate different signalling pathways on their ability to mimic cytokine-stimulated effects. As fMet-Leu-Phe and thrombin have been reported to stimulate phospholipase A₂ secretion from neutrophils [23] and platelets

[42], respectively, and both agonists activate phosphoinositide hydrolysis in these cells [24,25,47,48], we investigated the possible role of this important signalling cascade in phospholipase A₂ release from mesangial cells. Addition of angiotensin II, a potent activator of phosphoinositide turnover in mesangial cells [26], had no effect on phospholipase A₂ release from the cells. Likewise, addition of PMA, an activator of protein kinase C, or addition of the Ca²⁺ ionophore A23187 had no effect on phospholipase secretion. Furthermore, neither IL-1 nor TNF stimulated inositol polyphosphate generation and Ca²⁺ mobilization in mesangial cells (J. Pfeilschifter, unpublished work). This clearly shows that the bifurcating inositol lipid pathway does not contribute to the cytokine-stimulated phospholipase A₂ secretion from mesangial cells. Likewise, the addition of the cell-permeant cGMP analogue, Bt₂cGMP, did not stimulate phospholipase A₂ secretion, thus excluding cGMP as a possible messenger. In contrast, activation of the adenylate cyclase system consistently enhanced phospholipase A₂ release from mesangial cells. The time course of forskolin-stimulated phospholipase A₂ secretion as well as the pH- and Ca²⁺-dependence of the released enzyme were similar to those of the IL-1- and TNF-induced enzyme [13]. Furthermore, it has recently been demonstrated that cAMP mediates phospholipase A₂ secretion from vascular smooth-muscle cells [49]. From these results it is tempting to speculate that cAMP is the messenger that mediates IL-1- and TNF-stimulated phospholipase A₂ secretion. However, several lines of evidence argue against this suggestion. (a) Neither IL-1 β nor TNF stimulated any increase of cAMP in mesangial cells, irrespective of whether the cAMP-degrading phosphodiesterase was blocked or not. (b) The non-competitive adenylate cyclase inhibitor 2',5'-dideoxyadenosine [31–33], which has been shown to block PG-stimulated cAMP accumulation and phospholipase A₂ secretion in mesangial cells, did not inhibit IL-1 β - or TNF-stimulated phospholipase A₂ secretion. (c) Forskolin and IL-1 β or TNF synergistically interact to stimulate phospholipase A₂ secretion over the whole dose-response range, suggesting that IL-1 β and TNF use a messenger different from that for the forskolin-stimulated cAMP accumulation. Taken together, although cAMP is able to mimic IL-1- and TNF-induced phospholipase A₂ release from mesangial cells, the reported data provide strong evidence that cAMP is not the messenger that mediates IL-1- and TNF-stimulated enzyme secretion. In contrast with our results, Shirakawa *et al.* [50] showed that cAMP analogues and cAMP-inducing agents can replace IL-1 in the induction of interleukin 2 receptors on lymphocytes as well as in phytohaemagglutinin-induced murine thymocyte proliferation. Those authors also demonstrated that IL-1 stimulates a rapid and marked synthesis of cAMP in the IL-1 target cells. Activation of adenylate cyclase and cAMP-dependent protein kinase has also been suggested to play a role in IL-6-gene activation by TNF and IL-1 in fibroblasts [51,52]. In the mouse pituitary cell line AtT-20, IL-1 itself has no effect on cAMP, but IL-1 potentiates the forskolin-stimulated secretion of β -endorphin in a way similar to the synergistic interaction of IL-1 and forskolin in phospholipase secretion reported in the present paper [53]. In Jurkat T cells IL-1 does not modify the intracellular levels of cAMP and cGMP [54]. Obviously, IL-1 and TNF stimulate different signalling pathways in a tissue-selective manner. Recently, Burch *et al.* [55] reported that IL-1 stimulates prostaglandin synthesis and subsequent cAMP accumulation in Swiss 3T3 fibroblasts. A similar chain of events might also occur in mesangial cells, as IL-1 and TNF have been shown to stimulate PGE₂ synthesis [12–14], and PGE₂ in turn is known to stimulate adenylate cyclase in the cells [27]. However, the potent cyclo-oxygenase inhibitors indomethacin (Fig. 2) and diclofenac [28] completely abolished IL-1- and TNF-stimulated prostaglandin synthesis without affecting phospho-

lipase A_2 release, thus excluding prostaglandins as the mediators of cytokine-induced enzyme secretion from mesangial cells. Obviously, the amounts of prostaglandins formed in response to IL-1 or TNF are far below the concentrations needed to stimulate cAMP formation in mesangial cells. Furthermore, angiotensin II, which is a potent stimulator of prostaglandin synthesis in mesangial cells [56], has no effect on phospholipase A_2 release (Table 1). Lovett and colleagues [57] reported that IL-1 activates protein kinases in cultured glomerular mesangial cells. The kinase activation resulted in the phosphorylation of three predominant proteins. Specific tyrosine phosphorylation occurred at a 46 kDa protein, whereas a 55 kDa protein and a 20 kDa protein were phosphorylated on serine residues. In this connection it is noteworthy that different protein kinase inhibitors such as H-8, staurosporine, K-252a and amiloride were able to block IL-1- and TNF-induced phospholipase A_2 secretion from mesangial cells (Fig. 4, Table 4). With regard to the paper by Lovett *et al.* [57], a protein tyrosine kinase would be an attractive candidate for mediating phospholipase A_2 release from mesangial cells. Work is needed to test this hypothesis and may help to design effective therapeutic compounds to counteract the inflammatory effects of extracellular phospholipase A_2 . The pathophysiological significance of cAMP-mediated phospholipase A_2 release from glomerular mesangial cells and its possible role in the pathogenesis of glomerulonephritis remain to be elucidated.

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