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Tetranactin inhibits interleukin 1β and cAMP induction of group II phospholipase A_2 in rat renal mesangial cells

Gaby Walker [a], Dieter Kunz [a], Werner Pignat [b], Irmgard Wiesenberg [b], Henk Van den Bosch [c], Josef Pfeilschifter [a,*]

[a] *Department of Pharmacology, Biozentrum, Universität Basel, Klingelbergstraße 70, 4056 Basel, Switzerland*
[b] *Research Department, Pharmaceuticals Division, Ciba-Geigy Ltd., 4002 Basel, Switzerland*
[c] *Centre for Biomembranes and Lipid Enzymology, Padualaan 8, 3584 CH Utrecht, Netherlands*

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Abstract

Renal mesangial cells express secretory phospholipase A_2 in response to two principal classes of activating signals that may interact in a synergistic fashion. These two groups of activators comprise inflammatory cytokines, such as interleukin 1β, and agents that elevate cellular levels of cAMP. Treatment of mesangial cells with tetranactin, a cyclic antibiotic produced by *Streptomyces aureus* with a molecular structure similar to cyclosporin A inhibits interleukin 1β- and cAMP-dependent group II phospholipase A_2 secretion in a dose-dependent manner with IC_{50} values of 43 and 33 nM, respectively. However, tetranactin does not directly inhibit group II phospholipase A_2 activity. Western blot analyses of mesangial cell supernatants reveal that the inhibition of phospholipase A_2 activity is due to suppression of phospholipase A_2 protein synthesis. This effect is preceded by the reduction of phospholipase A_2 mRNA steady-state levels as shown by Northern blot analyses of total cellular RNA isolated from stimulated mesangial cells. Thus, tetranactin is a potent inhibitor of group II phospholipase A_2 expression in cytokine- and cAMP-stimulated mesangial cells and represents a new class of group II phospholipase A_2 inhibitors with IC_{50} values in the low nanomolar range. This compound may be useful in the therapy of diseases associated with increased group II phospholipase A_2 secretion.

Keywords: Phospholipase A_2; Interleukin 1β; cAMP; Tetranactin; Renal mesangial cell

1. Introduction

Phospholipase A_2 comprises a group of lipolytic enzymes that specifically release fatty acids, often arachidonic acid, from the sn-2 position of membrane phospholipids for production of important lipid mediators, such as eicosanoids and platelet-activating factor (Van den Bosch, 1980; Glaser et al., 1993). Arachidonic acid and its numerous metabolites act as intracellular and intercellular messengers contributing to normal cellular physiology by modifying the activity of intracellular enzymes and ion channels. It has become evident that phospholipase A_2 is a heterogenous family of enzymes that can be classified into two classes: high molecular weight phospholipase A_2 of 60–110 kDa, also referred to as cytosolic phospholipase A_2 and low molecular weight phospholipase A_2 of 14 kDa referred to as secretory phospholipase A_2. Members of both classes from human and rat sources have been cloned (Glaser et al., 1993; Dennis, 1994). Based on their primary structure, the secretory phospholipase A_2 is further classified into two groups (Heinrikson et al., 1977). Mammalian group I phospholipase A_2 comprises the pancreatic type of phospholipase A_2 whereas mammalian group II phospholipase A_2 is synthesized and secreted from many cell types and is believed to play a role in the initiation and propagation of inflammatory processes (for review, see Pruzanski and Vadas, 1991).

Among the cell types present in the glomerulus, mesangial cells are being increasingly recognized as a major determinant in the regulation of the glomerular filtration rate and blood flow. The cells do not only respond to vasoactive substances thought to be involved in the regulation of renal filtration, but they have themselves the capacity to produce considerable amounts of such mediators upon stimulation with cytokines and agents known to

* Corresponding author. Tel.: (41) (61) 2672223; fax: (41) (61) 2672208; e-mail: wittker@ubaclu.unibas.ch

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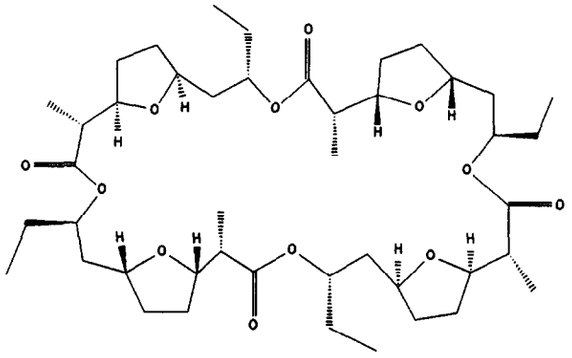

Fig. 1. Structure of tetranactin.

elevate intracellular cAMP levels (for review, see Pfeilschifter, 1994).

Two potent proinflammatory cytokines, interleukin 1β and tumor necrosis factor α, have been shown to induce the synthesis and secretion of group II phospholipase A_2 in mesangial cells (Pfeilschifter et al., 1989a; Schalkwijk et al., 1991) and in a variety of other cell types (for review, see Pfeilschifter, 1995). The cytokine effect is blocked by actinomycin D and cycloheximide, thus demonstrating a requirement for both transcription and de novo protein synthesis. This is confirmed by the observation that interleukin 1β and tumor necrosis factor α increase the level of mRNA for group II phospholipase A_2 in these cells (Mühl et al., 1991; Nakazato et al., 1991). We have demonstrated an increased secretion of group II phospholipase A_2 in rat vascular smooth muscle cells (Pfeilschifter et al., 1989b) and rat mesangial cells (Pfeilschifter et al., 1990, 1991) in response to cAMP-elevating agents. Furthermore, forskolin, an activator of adenylate cyclase, synergistically interacts with interleukin 1β to increase group II phospholipase A_2 mRNA levels in mesangial cells (Mühl et al., 1992).

Tetranactin is a hydrophobic cyclic antibiotic (Fig. 1) produced by *Streptomyces aureus* with a molecular structure related to that of cyclosporin A. As for cyclosporin A, strong immunosuppressive activity has been reported for tetranactin (Tanouchi and Shichi, 1988; Teunissen et al., 1992). Tetranactin has been shown to block the proliferation of T-lymphocytes as well as the generation of cytotoxic T-lymphocytes and activated killer cells (Callewaert et al., 1988). In view of the structural similarity of cyclosporin A and tetranactin, it is likely that the pharmacological activities of these compounds may be mediated by similar mechanisms. Previously, we have shown that cyclosporin A inhibits interleukin 1β- and tumor necrosis factor α-stimulated group II phospholipase A_2 secretion and prostaglandin synthesis (Pfeilschifter et al., 1989c).

The aim of the present work was to study the targets of action of tetranactin in renal mesangial cells and to evaluate its potential modulation of group II phospholipase A_2 expression. We report that tetranactin potently inhibits interleukin 1β- and cAMP-dependent induction of group II phospholipase A_2 by suppression of phospholipase A_2 protein and mRNA steady-state levels.

2. Materials and methods

2.1. Cell culture

Rat mesangial cells were cultured as described previously (Pfeilschifter and Vosbeck, 1991). In a second step, single cells were cloned by limited dilution using 96-microwell plates. Clones with apparent mesangial cell morphology were used for further processing. The cells exhibited the typical stellate morphology. Moreover, there was positive staining for the intermediate filaments desmin and vimentin, which are considered to be specific for myogenic cells, positive staining for Thy 1.1 antigen, negative staining for the factor VIII-related antigen and cytokeratin, excluding endothelial and epithelial contamination, respectively. The generation of inositol trisphosphate upon activation of the angiotensin II AT_1 receptor was used as a functional criterion for characterizing the cloned cell line. The cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and bovine insulin at 0.66 units/ml (Sigma). For the experiments, passages 8–16 of mesangial cell were used. For the labeling of newly synthesized proteins, 80 μCi of [^{35}S]methionine were added to 2 ml of methionine-free culture medium and cells were incubated for 6–8 h.

2.2. Phospholipase A_2 assay

Phospholipase A_2 activity was determined with [1-^{14}C]oleate-labeled *Escherichia coli* as substrate as described previously (Märki and Franson, 1986). The substrate was prepared by growing *E. coli* in the presence of [1-^{14}C]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-^{14}C]oleate was in the sn-2 position of the phospholipids. Assay mixtures (1.0 ml) contained 100 mM Tris–HCl buffer (pH 7.0), 1 mM $CaCl_2$, 2.5×10^8 [1-^{14}C]oleate-labeled *E. coli* (5 nmol of phospholipids, 5000–8000 c.p.m.) and the enzyme to be tested at a dilution producing less than 5% 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-heptan/1 M-H_2SO_4 (40 : 10 : 1, v/v/v) 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 × 0.5 cm). Free [1-^{14}C]oleic

acid was quantitatively eluted with 1 ml of ethylacetate. Radioactivity was determined in a scintillation counter. Phospholipase A_2 activity is expressed as [1-^{14}C]oleate radioactivity released by 100 μl of cell culture supernatant.

Inhibition of phospholipase A_2 activity was determined in assay mixtures containing enzyme extracted from human polymorphonuclear leukocytes (Märki and Franson, 1986). Percent inhibition relative to controls (absence of inhibitor) was calculated after correcting for blank hydrolysis determined in the absence of enzyme.

2.3. Immunoprecipitation

For the immunoprecipitation, 2 ml of culture supernatant were used containing proteinase – inhibitors at a final concentration of 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml leupeptin and 20 μM pepstatin. 100 μl of a 1 : 1 slurry of a specific monoclonal antibody (Aarsman et al., 1989; De Jong et al., 1987) coupled to protein-G-Sepharose were added and incubated overnight at 4°C. Immunoprecipitated proteins were analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis (15% polyacrylamide gel) as described previously (Huwiler et al., 1995).

2.4. Northern blot analysis

Confluent mesangial cells were washed twice with phosphate-buffered saline (PBS) and incubated in Dulbecco's modified Eagle's medium (DMEM), supplemented with 0.1 mg/ml of fatty acid-free bovine serum albumin, with or without agents for the indicated time periods. Cells were washed twice with PBS and harvested using a rubber policeman. Total cellular RNA was extracted from the cell pellets using the guanidinium thiocyanate/cesiumchloride method (Sambrock et al., 1989). Samples of 20 μg RNA were separated on 1% agarose gels containing 0.66 M formaldehyde prior to the transfer to gene screen membranes (New England Nuclear). After UV cross-linking and prehybridization for 2 h, the filters were hybridized for 16–18 h to a ^{32}P-labeled *Eco*RI/*Hin*dIII-cDNA insert from p139–1 coding for rat group II phospholipase A_2 (Van Schaik et al., 1993). To correct for variation in RNA amount, the phospholipase A_2 probe was stripped with boiling 0.1 × sodium chloride/sodium citrate buffer (SSC)/0.1% sodium dodecylsulfate (SDS) (Sambrock et al., 1989) and the blots were rehybridized to the ^{32}P-labeled *Bam*HI/*Sal*I cDNA insert from clone pEX 6 coding for human β-actin. DNA-probes (0.5–1 × 10^6 dpm/ml) were radioactively labeled with ^{32}P-dATP by random priming (Boehringer Mannheim). Hybridization reactions were performed in 50% (v/v) formamide, 5 × SSC, 5 × Denhardt's solution, 1% (w/v) SDS, 10% (w/v) dextran sulfate and 100 μg/ml salmon sperm DNA (Sambrock et al., 1989). Filters were washed 3 × in 2 × SSC/0.1% SDS at room temperature for 15 min, and then in 2 × in 0.2 × SSC/1% SDS at 65°C for 30 min. Filters were exposed for 24–48 h to Kodak X-Omat XAR-film using intensifying screens.

2.5. Statistics

Statistical analysis was done by one-way analysis of variance (ANOVA). For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons according to Bonferroni.

2.6. Materials

Recombinant human interleukin 1β was generously supplied by Dr. C. Rordorf (Ciba-Geigy, Basel, Switzerland). Tetranactin was kindly provided by Chugai Pharmaceutical (Tokyo, Japan). The cDNA-clone pEX 6, coding for human β-actin was a gift from Dr. U. Aebi (Basel, Switzerland). [1-^{14}C]oleic acid and [^{32}P]dATP (spec. act. 3000 Ci/mmol) were from Amersham (Dübendorf, Switzerland). Nylon membranes (Gene Screen) were purchased from DuPont de Nemours International (Regensdort, Switzerland). Cell culture media and nutrients were from Gibco BRL (Basel, Switzerland) and other chemicals used were either from Merck (Darmstadt, Germany) or Fluka (Basel, Switzerland).

3. Results

Incubation of mesangial cells for 24 h with interleukin 1β (2 nM) increases phospholipase A_2 activity secreted by mesangial cells by approximately 14-fold as shown in Table 1. Treatment of the cells with the membrane-permeable cAMP analogue, N^6, 0-2′-dibutyryladenosine 3′,5′-phosphate (Bt_2 cAMP, 10 mM) dramatically induces phospholipase A_2 activity in mesangial cells up to 22-fold

Table 1
Effects of tetranactin on interleukin 1β-stimulated phospholipase A_2 secretion from mesangial cells

Addition	Phospholipase A_2 secretion (cpm/100 μl)
Control	814 ± 64 [a]
Interleukin 1β (2 nM)	10890 ± 370
Interleukin 1β (2 nM) + tetranactin (1 μM)	384 ± 38 [a]
Interleukin 1β (2 nM) + tetranactin (100 nM)	1308 ± 48 [a]
Interleukin 1β (2 nM) + tetranactin (10 nM)	10013 ± 758
Interleukin 1β (2 nM) + tetranactin (1 nM)	12218 ± 410

Identical numbers of mesangial cells were incubated with indicated concentrations of interleukin 1β and tetranactin for 24 h. Thereafter, the medium was withdrawn and phospholipase A_2 activity determined as described in Materials and methods. Cell toxicity of tetranactin was excluded by a sensitive dye assay (MTT test). Results are means ± S.E. ($n = 4$). [a] $P < 0.001$. Significant differences from interleukin 1β stimulation (ANOVA).

Table 2
Effects of tetranactin on cAMP-stimulated phospholipase A_2 secretion from mesangial cells

Addition	Phospholipase A_2 secretion (cpm/100 μl)
Control	891 ± 19 [a]
Bt_2 cAMP (10 mM)	19283 ± 660
Bt_2 cAMP (10 mM) + tetranactin (1 μM)	535 ± 34 [a]
Bt_2 cAMP (10 mM) + tetranactin (100 nM)	3491 ± 267 [a]
Bt_2 cAMP (10 mM) + tetranactin (10 nM)	20553 ± 362
Bt_2 cAMP (10 mM) + tetranactin (1 nM)	22443 ± 535 [b]

Identical numbers of mesangial cells were incubated with the indicated concentrations of Bt_2 cAMP and tetranactin for 24 h. Thereafter, the medium was withdrawn and phospholipase A_2 activity determined as described in Materials and methods. Cell toxicity of tetranactin was excluded by a sensitive dye assay (MTT test). Results are means ± S.E. ($n = 4$). [a] $P < 0.001$, [b] $P < 0.05$. Significant differences from Bt_2 cAMP stimulation (ANOVA).

as shown in Table 2. Simultaneous incubation of cells with interleukin 1β (2 nM) or Bt_2 cAMP (10 mM) together with increasing amounts of tetranactin ranging from 1 nM up to 1 μM dose-dependently inhibits phospholipase A_2 secretion from mesangial cells (Table 1, Table 2). Half-maximal inhibition of interleukin 1β- and Bt_2 cAMP-stimulated phospholipase A_2 activity is observed at 43 and 33 nM of tetranactin, respectively. At the lowest concentration of tetranactin used (1 nM), we observed a moderate but significant increase of Bt_2 cAMP-induced phospholipase A_2 secretion (Table 2). Presently, we have no explanation for this effect but it should be noted that low concentrations of glucocorticoids also potentiate phospholipase A_2 secretion from stimulated mesangial cells (Pfeilschifter et al., 1993), whereas at higher concentrations glucocorticoids potently suppress phospholipase A_2 expression in the cells (Pfeilschifter, 1995).

Tetranactin does not inhibit purified phospholipase A_2 from human polymorphonuclear leukocytes up to concentrations of 10 μM, when assayed at a substrate concentration of 5 μM phospholipid (data not shown), thus, suggesting that tetranactin interferes either with the expression of phospholipase A_2 or its secretion from mesangial cells. For comparison, we also evaluated the effect of cyclosporin A on interleukin 1β- and Bt_2 cAMP-induced phospholipase A_2 secretion. Half-maximal inhibition is observed at 3.2 μM and 9.2 μM for interleukin 1β- and Bt_2 cAMP-stimulated cells, respectively (data not shown). Thus, tetranactin is approximately two orders of magnitude more potent than cyclosporin A in inhibiting phospholipase A_2 secretion in mesangial cells.

Immunoprecipitation of newly synthesized phospholipase A_2 protein with a monoclonal antibody demonstrates that treatment of mesangial cells with interleukin 1β (2 nM) and Bt_2 cAMP (10 mM) dramatically upregulates phospholipase A_2 protein as shown in Fig. 2. Tetranactin alone does not induce phospholipase A_2 protein

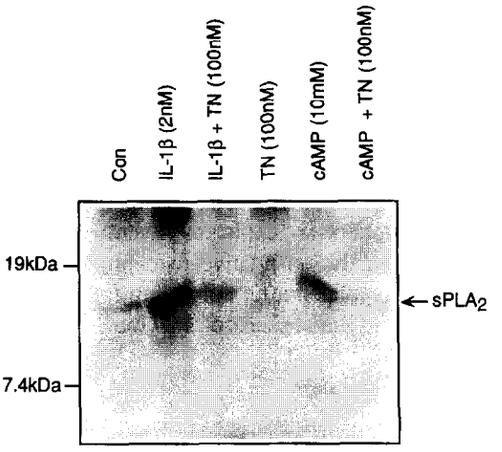

Fig. 2. Inhibition of interleukin 1β- and Bt_2 cAMP-stimulated group II phospholipase A_2 protein synthesis in mesangial cells by tetranactin. Mesangial cells were incubated for 16 h with vehicle (con.), Bt_2 cAMP (10 mM), interleukin 1β (2 nM) and tetranactin (TN, 100 nM) as indicated in the presence of [^{35}S]methionine. Thereafter, group II phospholipase A_2 was immunoprecipitated and samples were analyzed as described in Materials and methods.

synthesis. However, coincubation of interleukin 1β (2 nM) and Bt_2 cAMP (10 mM) with tetranactin (100 nM) suppresses synthesis of phospholipase A_2 protein (Fig. 2).

In order to assess whether the suppression of phospholipase A_2 protein synthesis by tetranactin is associated with changes in the mRNA levels coding for group II phospholipase A_2, we performed Northern blot analyses of total cellular RNA isolated from mesangial cells stimulated for 24 h with interleukin 1β or Bt_2 cAMP in the absence

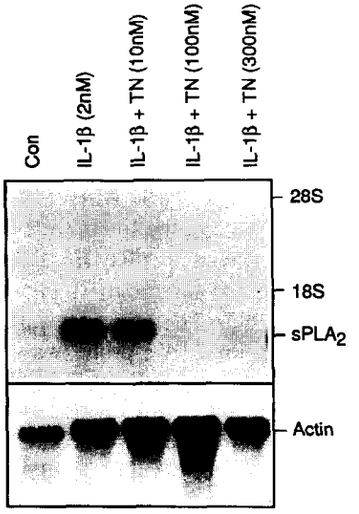

Fig. 3. Inhibition of interleukin 1β-stimulated induction of group II phospholipase A_2 mRNA in mesangial cells by tetranactin. Mesangial cells were incubated for 24 h with vehicle (con.), interleukin 1β (2 nM) or interleukin 1β plus the indicated concentrations of tetranactin (TN). Total RNA (20 μg) was successively hybridized to ^{32}P-labeled group II phospholipase A_2 and β-actin cDNA probes as described in Materials and methods.

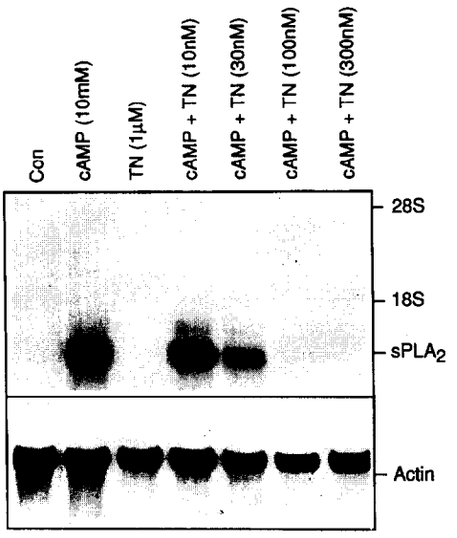

Fig. 4. Inhibition of Bt_2 cAMP-stimulated induction of group II phospholipase A_2 mRNA in mesangial cells by tetranactin. Mesangial cells were incubated for 24 h with vehicle (con.), Bt_2 cAMP (10 mM) or Bt_2 cAMP plus the indicated concentrations of tetranactin (TN). Total RNA (20 μg) was successively hybridized to ^{32}P-labeled group II phospholipase A_2 and β-actin cDNA probes as described in Materials and methods.

or presence of tetranactin in concentrations ranging from 10 nM up to 300 nM. As shown in Fig. 3, tetranactin dose-dependently downregulates the interleukin 1β-induced phospholipase A_2 mRNA steady-state levels. At a concentration of 100 nM, tetranactin completely inhibits phospholipase A_2 mRNA formation. Similarily, formation of Bt_2 cAMP-induced phospholipase A_2 mRNA steady-state levels is suppressed by the action of tetranactin in a dose-dependent fashion (Fig. 4).

4. Discussion

Cyclosporin A and FK 506 are potent immunosuppressive drugs clinically used for the prevention of graft rejection following organ transplantation. These drugs bind to intracellular receptor proteins, collectively termed immunophillins, that have peptidyl-prolyl *cis-trans* isomerase activity. The complex between drug and its cognate immunophilin inhibits the intracellular Ca^{2+}-dependent protein phosphatase calcineurin. The activity of calcineurin is required for the activation and nuclear translocation of a subunit of the transcription factor NF-AT, known to be important in the transcriptional regulation of T-cell-specific genes. Thereby, the drugs suppress Ca^{2+}-dependent T-cell responses, such as T-cell receptor-mediated transcription of interleukin 2 (for review, see Schreiber and Crabtree, 1992). The therapeutic application of these drugs, however, is limited by considerable side-effects, notably nephrotoxicity and neurotoxicity (Mason, 1989). Numerous reports on nephrotoxic activities of immunosuppressive drugs, such as cyclosporin A, suggest that these effects are due to its action on renal cells, thus, interfering with important regulatory mechanisms in the kidney.

Among the cell types present in the glomerulus, mesangial cells are being increasingly recognized as a major determinant in the regulation of glomerular filtration rate and blood flow. Mesangial cells are an abundant source of vasoactive substances, such as prostaglandines or platelet-activating factor in the kidney. These may act as intracellular and intercellular messengers contributing to normal cellular physiology by modifying the activity of intracellular enzymes and ion channels (for review, see Pfeilschifter, 1994, 1995).

Tetranactin is a member of the group of polynactins, hydrophobic cyclic antibiotics produced by *Streptomyces aureus*. The chemical structure is similar to that of the cyclic polypeptide cyclosporin A, suggesting that tetranactin may have comparable properties. Strong immunosuppressive activity was described for tetranactin (Callewaert et al., 1988; Teunissen et al., 1992). The authors demonstrated that tetranactin – with a potency comparable to cyclosporin A – blocks the initiation of interleukin 2-dependent proliferation of human T-lymphocytes. Furthermore, the generation of cytotoxic T-lymphocytes and activated killer cells in mixed lymphocyte cultures was suppressed.

We report here that tetranactin inhibits the interleukin 1β- and cAMP-dependent induction of group II phospholipase A_2 expression in mesangial cells. Stimulation of the cells with interleukin 1β or Bt_2 cAMP strongly induces group II phospholipase A_2 activity secreted by mesangial cells. The IC_{50} values for inhibition are in the nanomolar range, suggesting that tetranactin is highly potent in exerting its inhibitory effects in mesangial cells. The reduction of group II phospholipase A_2 release is due to decreased synthesis of group II phospholipase A_2 by mesangial cells as shown by in vivo labeling experiments with [^{35}S]methionine (Fig. 2). Northern blot analysis strongly suggests that the observed reduction of group II phospholipase A_2 protein synthesis is the consequence of decreased steady-state levels of group II phospholipase A_2 mRNA. Whether this phenomenon is caused by a decreased transcriptional activity of the group II phospholipase A_2 gene or a decreased stability of group II phospholipase A_2 mRNA or a combination of both remains to elucidated. In this context, it is worth mentioning that tetranactin also inhibits gene transcription of inducible nitric oxide synthase (G. Walker, D. Kunz, I. Wiesenberg and J. Pfeilschifter, unpublished observation), another potent proinflammatory mediator produced in renal mesangial cells (Pfeilschifter, 1994). Again, tetranactin is approximately 20–100-fold more potent than cyclosporin A in doing so. It will be a fascinating task to unravel the signalling cascades affected by tetranactin and to pinpoint in more detail the level of interaction with the induction of group II phospholipase A_2 and other inflammatory mediators in mesangial cells.

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