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Cyclic AMP increases rat inhibitor of apoptosis protein (RIAP1) mRNA in renal mesangial cells

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Background. The cyclic adenosine 3′,5′-monophosphate (cAMP) pathway plays a central role in the regulation of cell proliferation, differentiation and apoptosis. Cyclic AMP has been identified as a bifunctional regulator of apoptosis. The inhibitor of apoptosis proteins (IAP) regulates apoptosis by directly inhibiting distinct caspases.

Methods. Expression levels of rat IAP (RIAP)-1 were investigated by RNase protection assay in rat mesangial cells after stimulation with diverse agents that modulate cellular levels of cAMP.

Results. Rat mesangial cells up-regulated RIAP1 mRNA levels after cAMP stimulation. Membrane-permeable cAMP analogs, as well as cAMP production in response to the β-adrenergic receptor agonist salbutamol caused a large increase in RIAP1 mRNA level, which could be inhibited by the protein kinase A inhibitors H89 and Rp-cAMPS, or by the nuclear factor-κB (NF-κB) inhibitor BAY117085. Inhibition of phosphodiesterase type IV by denbufyllin or rolipram potentiated the cAMP-mediated increase in RIAP1 mRNA. In contrast, the cyclic guanosine 3′,5′-monophosphate (cGMP) analog Bt_2cGMP did not affect the RIAP1 mRNA level.

Conclusions. These data establish, to our knowledge for the first time, that RIAP1 mRNA levels are regulated by the cAMP-signaling pathway and suggest potential new avenues of therapy to modulate apoptosis.

Regulation of cell homeostasis in multicellular organisms involves the generation of new cells as well as removal of unwanted or damaged cells by cell death. There exists in all cells the necessary equipment for programmed cell death (apoptosis), which usually is suppressed when a cell is doing well but allows rapid cell death when required. Caspases are the major executioners of apoptosis, as they initiate a self-amplifying cascade of autoactivation as well as cleave many proteins to ensure cellular homeostasis [1, 2]. A powerful barrier against uncontrolled activation of caspases is the inhibitor of apoptosis (IAP) proteins that directly bind to and inhibit caspases and thus block intrinsic and extrinsic pathways of programmed cell death. IAPs were first described in baculovirus as proteins that inhibit cell death and thus allow the virus to replicate efficiently in infected cells. These evolutionary conserved proteins contain tandem repeats of domains, denoted as baculovirus IAP repeats (BIR domains). Some of them also contain a C-terminal RING zinc-finger domain. At least seven different mammalian IAPs, including X-chromosome-linked IAP (XIAP), cellular inhibitor of apoptosis protein 1 (cIAP-1), cIAP-2, neuronal apoptotic inhibitory protein (NAIP), ML-IAP, apollon and survivin are known to exhibit anti-apoptotic activity [1].

It is not yet fully understood how these proteins are regulated in cells and tissues. Some IAPs are known to be regulated by nuclear factor-κB (NF-κB), and thus might intersect with the apoptotic pathway and explain, at least partially, the anti-apoptotic effects of NF-κB [3–5]. Moreover, IAP regulation by nitric oxide [6] and hypoxia [7] has been observed to involve non-conventional pathways of gene transcription. Glucocorticoids also have been reported to induce IAP expression or to prevent IAP degradation, and thus suppress inflammatory responses and prevent cells from undergoing apoptosis [8, 9]. This article reports that cyclic 3′,5′-monophosphate (cAMP) up-regulates cIAP1 (RIAP1 in the rat) in rat mesangial cells, which may strengthen their commitment to live and subsequently alter the course of inflammatory glomerular diseases.

METHODS

Materials

RNases A, RNase T1, RNA polymerase T3 and T7 were from Roche Molecular Biochemicals (Mannheim, Germany). N^6,$O^{2'}$-dibutyryl-cAMP (Bt_2cAMP), N^6,$O^{2'}$-dibutyryl-cGMP (Bt_2cGMP), 8-bromo-cAMP (Br-cAMP), 8-(4-chlorophenylthio)-cAMP (8-CPTcAMP), forskolin, cholera toxin Inaba 569B, 3-isobutyl-1-methylxanthine (IBMX), pyrrolidine dithiocarbamate (PDTC) and guanidine isothiocyanate were purchased from Sigma (Dei-

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senhofen, Germany). Adenosine-3′,5′-cyclic monophosphorothioate (Rp-Isomer, triethylammonium salt), H89, Cilostamide, Rolipram, Denbufyllin and BAY117085 were purchased from Calbiochem (Bad Soden, Germany). RPMI 1640, Dulbecco's modified Eagle's medium (DMEM), cell culture supplements and fetal calf serum (FCS) were from Gibco (Eggenstein, Germany). All other chemicals were of the highest grade of purity commercially available.

Cell culture and cell treatment

Rat renal mesangial cells were cultured and characterized as described previously [10]. In a second step, single cells were cloned by limited dilution of 96-well plates [11]. For the experiments in this study passages 13 to 23 were used.

Cells were cultured in RPMI 1640 medium containing 10% FCS, penicillin (100 U/mL), and streptomycin (100 μg/mL). For all experiments, subconfluent cells were starved for 24 hours and exposed to the different substances in starvation medium (DMEM containing 0.1 mg/mL fatty acid-free BSA, penicillin 100 U/mL, and streptomycin 100 μg/mL).

Probe DNAs

All the cDNA fragments used as probes for RNase protection assays were cloned by polymerase chain reaction (PCR) using the following sequences as nucleotide primers, purchased from Life Technologies (Eggenstein, Germany):

Rat IAP1 probe: 5′-CTC ATG (GC)AC AAA ACT G(CT)C TCC-3′ was used as a 5′-primer and 5′-CT(AG) GG(AG) TA(GCT) A(AGC)C T(GT)T (CT)T(AG) TGC-3′ as a 3′-primer.

Rat β-actin probe: 5′-ATG GAT GA(CT) GAT ATC GC(TC) GCG-3′ was used as a 5′-primer and 5′-ATG GGG TAC TTC AGG GT(CG) AGG-3′ as a 3′-primer.

Rat GAPDH probe: 5′-CAT CAC CAT CTT CCA GGA GCG AG-3′ as a 5′-primer and 5′-GTT GTC ATA (CT)TT CTC (AG)TG GTT C-3′ as a 3′-primer.

RNA isolation and RNase protection analysis

RNA isolation was performed as described [6]. Twenty micrograms of total RNA from the different experimental time points of the cell culture experiments were used for RNase protection assays. DNA probes were cloned into the transcription vectors pBluescript II KS(+) (Stratagene, Heidelberg, Germany) or pCR2.1-TOPO (Invitrogen, Leek, Netherlands) and linearized. An anti-sense transcript (riboprobe) was synthesized in vitro using T3 or T7 RNA polymerase and [α^{32}P]UTP (800 Ci/mmol; Amersham, Freiburg, Germany). RNA samples were hybridized at 42°C overnight with 100,000 cpm of the riboprobe. Hybrids were digested with a mix of RNase A and T1 for one hour at 30°C. Protected fragments were separated on 5% acrylamide/8 mol/L urea gels and analyzed using a PhosphoImager (Fuji, Tokyo, Japan). All protection assays were carried out with at least three different sets of RNA from independent experiments.

Western blot analysis

Cells were cultured and incubated as indicated. Cell lysis and immunoblotting was performed as previously described [6].

RESULTS

Cyclic AMP increases RIAP-1 mRNA levels in mesangial cells

Mesangial cells were incubated with forskolin and after a period of eight hours there was a significant and time-dependent increase in RIAP1 mRNA levels (Fig. 1A). This response to forskolin was dose-dependent, with a threshold at 10 μmol/L forskolin. In the presence of 10 μmol/L forskolin, cAMP levels in mesangial cells increased 14-fold during the first hours and remained at an elevated concentration during at least 24 hours of incubation [12].

The diterpene forskolin is known to activate adenylate cyclase by direct stimulation of the catalytic submit of the enzyme. We next investigated the effects of the β-adrenoceptor agonist salbutamol and cholera toxin, two compounds that activate adenylate cyclase system by different mechanisms, on RIAP1 mRNA levels.

Salbutamol binds to β-adrenoceptors on the cell surface of mesangial cells [13] and activates adenylate cyclase via the stimulatory G-protein, G_s. Cholera toxin causes adenosine diphosphate (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 depicted in Figure 1B for cholera toxin and in Figure 1C for salbutamol, both agents enhanced RIAP1 mRNA levels in a time- and dose-dependent manner. In a further approach the membrane-permeant analogs of cAMP (Bt_2 cAMP, 8-Br-cAMP and 8-CPT-cAMP) were used to elevate the intracellular concentration of cAMP directly. Figure 1A clearly shows that all cAMP analogs increased RIAP1 mRNA levels in mesangial cells. In addition, the broad spectrum phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine and the selective inhibitors of phosphodiesterase 4, rolipram and denbufyllin, but not the selective inhibitor of phosphodiesterase 3, cilostamide (which increase cAMP by inhibiting the enzymes that degrade it), also moderately enhanced RIAP1 mRNA levels (data not shown) and a combination of IBMX with salbutamol resulted in a synergistic up-regulation of RIAP1 mRNA (Fig. 1C).

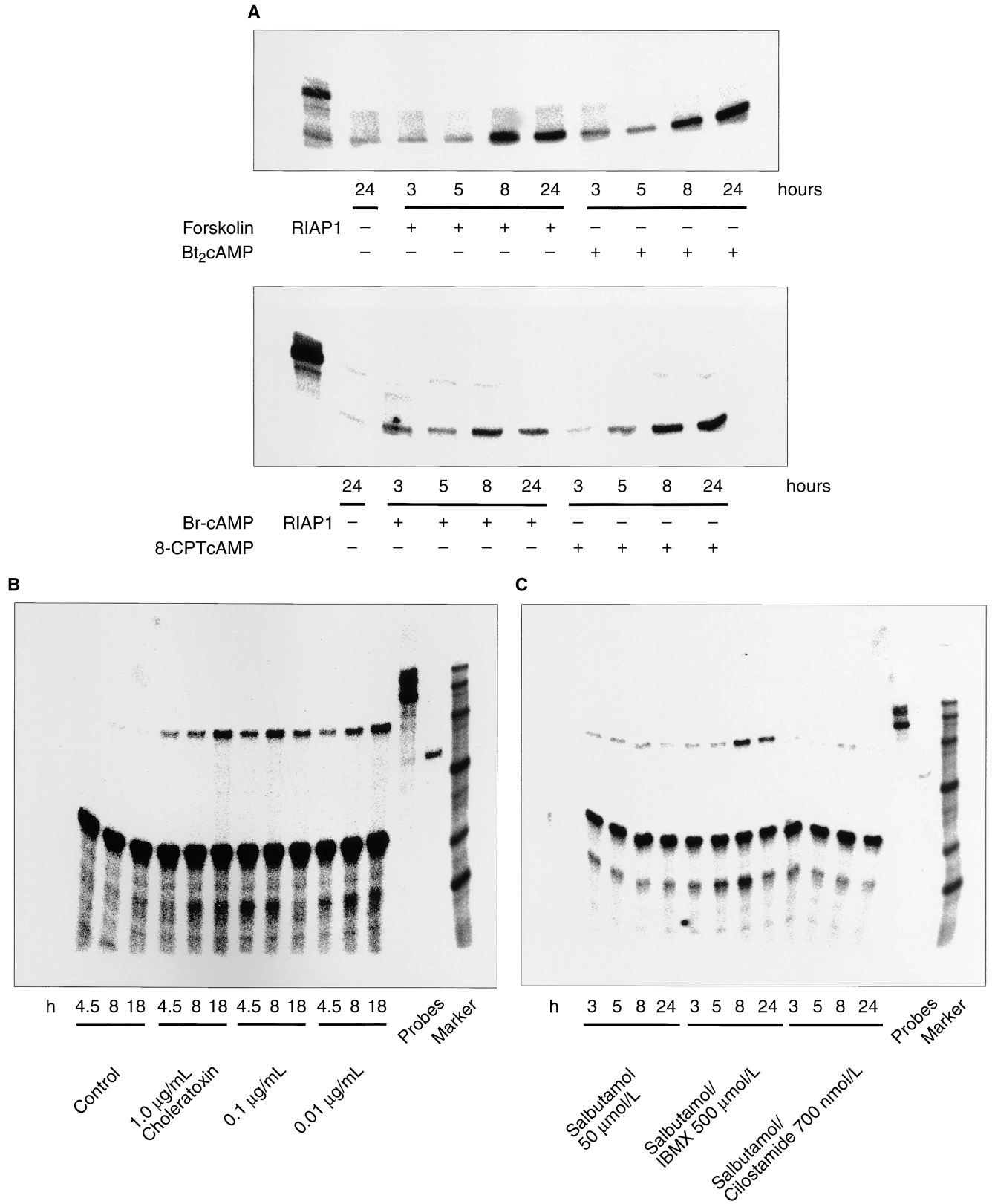

Fig. 1. Cyclic adenosine 3′,5′-monophosphate (cAMP) increases rat inhibitor of apoptosis protein (RIAP1) mRNA expression in renal mesangial cells. Rat renal mesangial cells were incubated for the indicated time periods with (*A*) forskolin (10 μg/mL), N^6,O^2-dibutyryl-cAMP (Bt_2cAMP, 1 mmol/L), 8-bromo-cAMP (Br-cAMP, 1 mmol/L, 8-(4-chlorophenylthio)-cAMP (8-CPTcAMP, 1 mmol/L) or with (*B*) cholera toxin (1.0 μg/mL, 0.1 μg/mL 0.01 μg/mL) or with (*C*) salbutamol (50 μmol/L) alone or in combination with 3-isobutyl-1-methylxanthine (IBMX, 500 μmol/L) or Cilostamide 700 nmol/L, as indicated. 20 μg total RNA were analyzed by RNase protection assay. The RIAP1 mRNA expression levels were assessed by PhosphorImager analysis of the radiolabeled gels. Representative RNase protection gels from three independent experiments are presented.

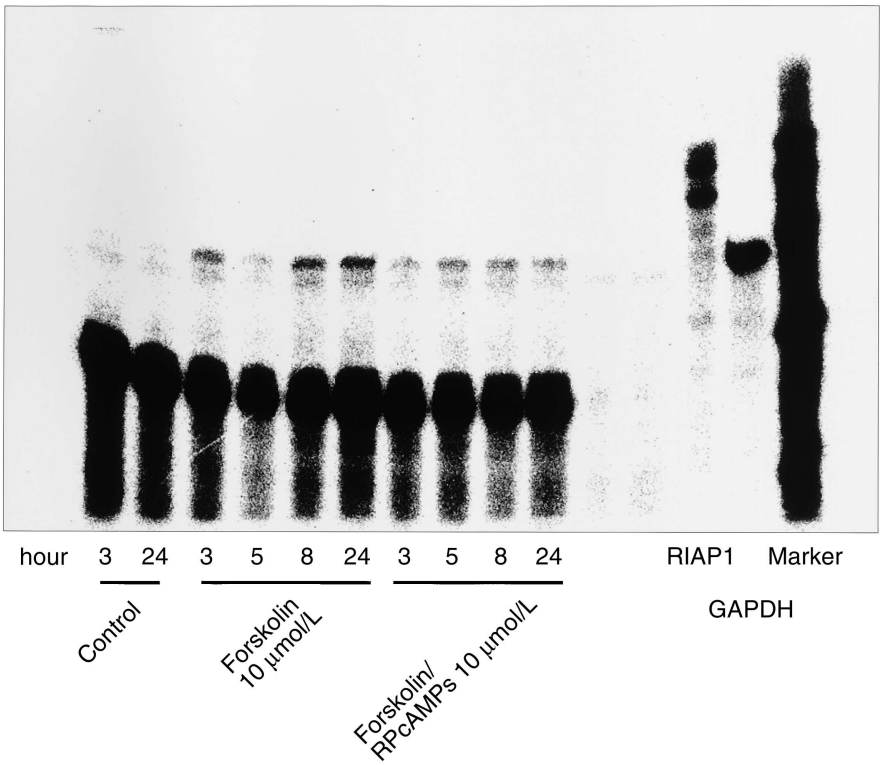

Fig. 2. Inhibition of protein kinase A (PKA) blocks RIAP1 mRNA expression in renal mesangial cells. Rat renal mesangial cells were incubated for the indicated time periods with forskolin (10 μmol/L) alone or in combination with RPcAMPS (10 μmol/L). A representative RNase protection gel out of three independent experiments is presented.

Cyclic GMP does not increase RIAP1 mRNA levels in mesangial cells

Incubation of mesangial cells with the membrane-permeable cyclic guanosine 3′,5′-monophosphate (cGMP) analog Bt_2 cGMP did not alter RIAP1 mRNA levels (data not shown). Furthermore, inhibition of soluble guanylate cyclase by the specific inhibitor NS 2028 had not effect on RIAP1 mRNA levels. (data not shown). Recently we have demonstrated that nitric oxide (NO) up-regulates RIAP1 mRNA in mesangial cells [6]. As it is well established that cAMP mediates expression of inducible nitric oxide synthase (iNOS) in rat mesangial cells [12, 14] and increases NO production by the cells, we evaluated whether the cAMP-induced RIAP1 expression is triggered by prior formation of nitric oxide. To this end mesangial cells were incubated with Bt_2 cAMP in the presence or absence of the nitric oxide synthase inhibitor L-N^G-monomethyl-L-arginine and determined RIAP1 expression. There was no significant alteration of RIAP1 expression by the NOS inhibitor (data not shown), suggesting that cAMP directly alters gene expression of RIAP1.

Inhibition of protein kinase A blocks RIAP1 mRNA expression

Increased formation of cAMP signals most cellular responses through activation of the cAMP-dependent protein kinase (PKA), which is a cytoplasmic heterotetramer of paired regulatory and catalytic subunits under basal conditions. Binding of cAMP causes the dissociation of the heterotetramer and liberates the catalytic subunits that translocate to the nucleus and alter gene expression by phosphorylating cAMP response element (CRE)-binding protein (CREB) [15]. To determine whether PKA activation triggers RIAP1 expression we used a specific inhibitor of PKA, the cyclic cAMP analog RpcAMPS [16]. As shown in Figure 2, RpcAMPS (10 μmol/L) caused a marked inhibition of forskolin-induced RIAP1 expression, thus confirming the crucial role for PKA in cAMP-induced RIAP1 expression. Surprisingly, BAY 117085, an inhibitor of IκB kinase and NF-κB signaling in mesangial cells, blocked forskolin-induced RIAP1 mRNA expression (Fig. 3). Although most Rel proteins (subunits that form the transcription factor NF-κB) have consensus recognition sites for PKA phosphorylation and cAMP has been shown to activate NF-κB in certain cell types [17], it has been clearly documented that cAMP does not activate NF-κB in mesangial cells [18, 19]. Obviously, pharmacological inhibitors do not always give satisfactory answers because many of these compounds are not highly specific and do not only inhibit their supposed target enzymes.

Cyclic AMP does not alter RIAP1 protein levels

Because the effect of nitric oxide on RIAP1 expression in mesangial cells was limited to mRNA regulation, with

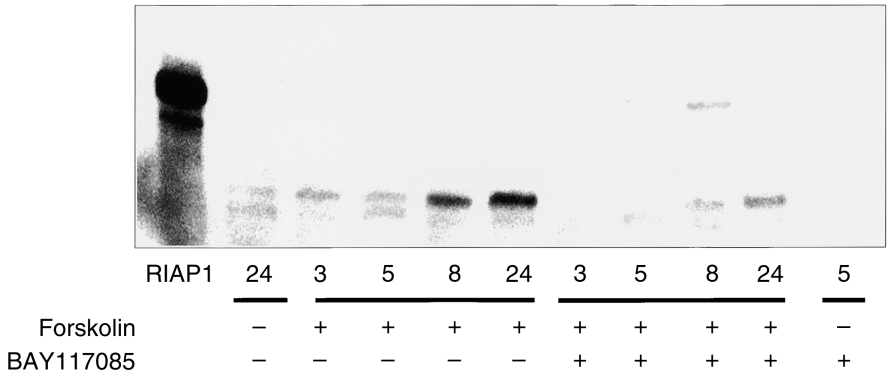

Fig. 3. BAY 117085 blocks RIAP1 mRNA expression in renal mesangial cells. Rat renal mesangial cells were incubated for the indicated time periods with forskolin (10 μmol/L) alone or in combination with BAY 117085 (30 μmol/L), as indicated. 20 μg total RNA were analyzed by RNase protection assay. The RIAP1 mRNA expression levels were assessed by PhosphorImager analysis of the radiolabeled gels. Representative RNase protection gels from three independent experiments are presented.

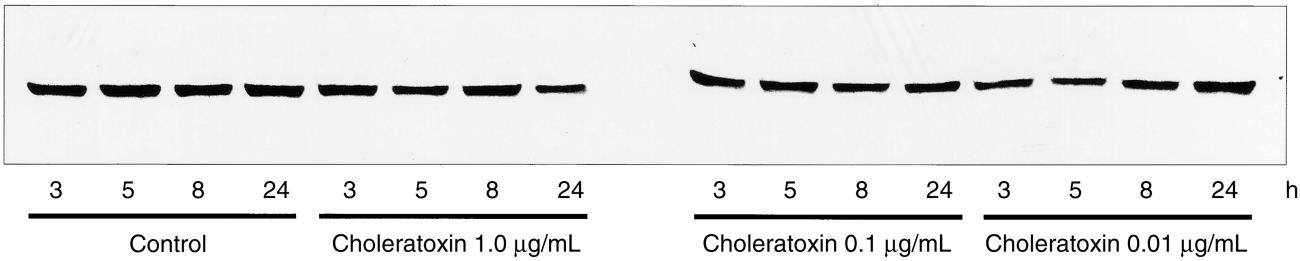

Fig. 4. Effect of cAMP on RIAP1 protein in renal mesangial cells. Rat renal mesangial cells were incubated for the indicated time periods with vehicle (control) or cholera toxin (1.0 μg/mL, 0.1 μg/mL, 0.01 μg/mL) for the indicated time periods. 50 μg total protein were analyzed by Western blot, followed by enhanced chemiluminescence detection and video densitometry. Each blot is representative of three similar experiments.

only marginally changes in RIAP1 protein levels, we next investigated the effects of cAMP on RIAP1 protein levels. Surprisingly, RIAP1 mRNA up-regulation by cholera toxin (Fig. 4) or forskolin (data not shown) did not result in RIAP1 protein up-regulation in mesangial cells, indicating that changes in RIAP1 mRNA do not necessarily translate into changes in protein levels.

DISCUSSION

Excessive proliferation of mesangial cells is a characteristic feature of many glomerular diseases caused by diverse immunological and non-immunological injuries. The initial phase of injury as well as resolution of hyperproliferative glomerular diseases have been shown to involve apoptosis of mesangial cells to avoid aberrant wound healing responses and fibrosis [2, 20]. In molecular terms, a family of proteases, the caspases, has been identified as a pivotal executioner of programmed cell death. Primarily activation of caspase 3 may mark a point of no return in the apoptotic cascade [1]. Therefore, it is not surprising that IAPs are ubiquitously expressed to halt the caspase cascade unless apoptosis is actually required. Expression of IAPs has been reported in human kidneys [21, 22] and recently regulation of IAP expression in mesangial cells in response to interleukin-1β (IL-1β) [6], tumor necrosis factor-α (TNFα) [6, 23] and, remarkably, NO [6] has been observed. Previous investigations suggested that IAP expression by cytokines such as TNFα, bacterial endotoxins and ionizing radiation occurs through NF-κB [3–5, 24, 25]. Our investigation found, to our knowledge for the first time, that the cAMP/PKA pathway is an alternative pathway of transcriptional regulation of IAP expression and may constitute a powerful protective agent in cells exposed to stress conditions. In this context, type I PKA has been reported to delay apoptosis in response to different stimuli in human neutrophils at a site upstream of caspase 3 [26], and expression of PKA inhibitor gene was found to abolish cAMP-mediated protection to endothelial barrier dysfunction [27]. Moreover, cAMP-derived signaling and gene expression inhibit apoptosis in macrophages [28] and vascular smooth muscle cells [29]. Several mechanisms have been proposed for the anti-apoptotic action of cAMP signaling, including cross-talk to the mitogen-activated protein kinase pathway [26], changes in intracellular Ca^{2+} mobilization, interference with cytoskeletal reorganization and expression of protective mediators like cyclooxygenase 2 [28]. We now provide convincing evidence that the expression of IAPs is regulated by cAMP and may provide a powerful brake to cellular apoptotic processes. Recently, a 3.5 kb fragment of the 5′-flanking region of the human c-IAP2 gene has been cloned, and several potential binding sites for diverse

transcription factors have been delineated [30]. The mechanism of transcriptional regulation of IAP genes by cAMP, however, remains to be elucidated. Another exciting avenue of study will be to analyze the mechanism and to identify the signal for the translational switch, initiating translation of locally docked but translationally inactive RIAP1 mRNA. This ability to induce the synthesis of IAP proteins would allow for long-lasting protection of cells or, at least, delay cell death. In this context it is important to note that cAMP also can trigger apoptosis in certain cells like human neuroblastoma cells [31] and, surprisingly, in mesangial cells [32]. Actually, cAMP-induced IAP expression may provide means to dampen the pro-apoptotic processes simultaneously initiated by this second messenger molecule and allow a finely tuned regulation of cell response. It is possible that elevation of cAMP concentrations by agonists such as β-adrenergic agents, prostaglandins, adrenomedulin, or other agents in glomerular mesangial cells or in invading immune cells may serve to delay programmed cell death in vivo in the course of different forms of glomerulonephritis [20].

Worth mentioning is that cAMP accumulation in T cells and other inflammatory cells is generally associated with inhibition of effector cell function. Early studies suggested that prostaglandin E_2 protects immature $CD4^+CD8^+$ (double positive) T lymphoblasts from apoptosis by a cAMP-mediated action [33]. Whether this involves IAP expression has not been evaluated but in the light of the present study this seems to be an intriguing hypothesis.

Furthermore, treatment with inhibitors of types III and IV phosphodiesterase have been reported to reduce macrophage accumulation and mesangial cell proliferation during the acute phase of anti-Thy 1.1 serum induced glomerulonephritis [34, 35], and type IV phosphodiesterase inhibition is effective in prevention and treatment of experimental crescentic glomerulonephritis [36]. The mechanism by which these clinically available phosphodiesterase inhibitors act has been ascribed to cAMP-mediated suppression of chemokines and cell adhesion molecules. It is tempting to speculate that phosphodiesterase inhibitors also alter IAP expression in vivo and thus may contribute to the anti-inflammatory and antifibrotic action of these drugs. Uncovering the precise regulation and action of IAPs should yield important information about the biological significance of these proteins in inflammation and may uncover new therapeutic targets for a number of diseases.

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