Independent regulation of cyclo-oxygenase 2 expression by p42/44 mitogen-activated protein kinases and Ca²⁺/calmodulin-dependent kinase

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5-Hydroxytryptamine (5-HT, 'serotonin') is a potent inducer of the early response gene cyclo-oxygenase 2 (Cox-2; prostaglandin G/H synthase) in mesangial cells. Protein kinase C (PKC), Ca²⁺-dependent enzymes and mitogen-activated protein kinase (p42/44 MAPK) have previously been shown to be essential modules of the signalling pathway leading from the pertussis-insensitive 5-HT_{2A} receptor to the induction of Cox-2 mRNA expression. In the present study, PKC activation was linked to the 5-HT-mediated phosphorylation and thus the activation of p42/44 MAPK: the inhibition of PKC by the specific inhibitor GF109203x prevented p42/44 MAPK activation. Ca²⁺/calmodulin-dependent (CaM) kinase II δ_2 was detected in mesangial

cells by Western blot analysis. The inhibition of CaM kinase by the inhibitors KN62 or KN93 led to a partial inhibition of 5-HT-induced Cox-2 mRNA expression and decreased basal, but not PMA-mediated, Cox-2 expression. The 5-HT-mediated activation of MAPK was not decreased by KN62 or KN93, excluding CaM kinase as a signalling module upstream of p42/44 MAPK. Taken together, these results indicate a modulatory involvement of CaM kinase in the regulation of 5-HT-mediated Cox-2 mRNA expression in addition to the main pathway that consists of the activation of PKC and p42/44 MAPK.

Key words: mesangial cells, protein kinase C, serotonin.

INTRODUCTION

Cyclo-oxygenases (Cox; prostaglandin G/H synthase), key enzymes of prostaglandin biosynthesis, exist in two isoforms, Cox-1 and Cox-2, which catalyse the same enzymic reaction but are differentially localized and regulated [1]. The regulation of mRNA levels is one of the main modes of regulation of the availability of Cox-2 and thus its activity [2,3]. Understanding the signalling pathways that control mRNA expression therefore relates to understanding how this biosynthetically important enzyme is regulated. Rat renal mesangial cells provide an appropriate model system for such studies. These are primary cells that can be cultivated over several passages without loss of function [4]. Multiple stimuli can induce Cox-2 expression in mesangial cells, among them mediators that bind to G-protein-coupled seven-transmembrane receptors such as endothelin [5], lysophosphatidic acid [6] or 5-hydroxytryptamine (5-HT, 'serotonin') [7,8]. 5-HT is stored in granules of platelets and can be released from activated platelets found in the kidney at sites of glomerular injury [9]. The induction of Cox-2 in mesangial cells by 5-HT will then contribute to an enhanced release of prostaglandin E₂, a vasodilating prostaglandin opposing the vasoconstrictor effects of 5-HT.

We have shown previously that 5-HT activates mesangial cells by binding to 5-HT_{2A} receptors that couple to pertussis toxin-insensitive G-proteins of the G_q family [8,10]. Phosphatidylcholine-specific phospholipase C, protein kinase C (PKC) and mitogen-activated protein kinase (p42/44 MAPK) were shown to be critical elements of the signalling pathway leading to the induction of Cox-2 mRNA expression initiated by 5-HT receptor binding [8]. These studies, however, have not yet connected activation of the 5-HT_{2A} receptor, PKC and MAPK.

Furthermore, pretreatment of mesangial cells with the Ca²⁺ chelator EGTA almost completely prevented the induction of the expression of Cox-2 mRNA and protein, indicating a substantial role for Ca²⁺-dependent enzymes in the signalling cascade [11,12]. The Ca²⁺/calmodulin-dependent protein kinases (CaM kinases) are important enzymes in Ca²⁺-mediated signal transduction systems [13,14]. In neuronal cells CaM kinases are involved in the induction of early response genes *c-fos*, *NGFI-A* and *NGFI-B* [15]. CaM kinases were also shown to have a role in the transcriptional regulation of *c-fos* expression in mesangial cells [16]. These kinases were thus also likely candidates for contributing to the induction of the early-response gene Cox-2. There is little experimental evidence linking the activation of CaM kinases to other signalling pathways. In vascular smooth-muscle cells, which have many features in common with mesangial cells [17,18], activation of CaM kinase II was related to an activation of MEK, a MAPK kinase [19]. It was therefore tempting to speculate that a similar mechanism might also be relevant for the Ca²⁺-dependent induction of Cox-2 mRNA in mesangial cells.

In the present study the role of CaM kinases in the gene induction of Cox-2 was investigated in mesangial cells and related to other signalling modules, namely PKC and MAPKs. We present evidence that the activation of CaM kinases and of MAPKs is independently linked to the 5-HT-mediated induction of Cox-2 mRNA in mesangial cells.

EXPERIMENTAL

Materials

Cell culture media consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine, penicillin

Abbreviations used: CaM, Ca²⁺/calmodulin-dependent; Cox-2, cyclo-oxygenase 2; CRE, cAMP-response element; CREB, cAMP-response element-binding protein; DMEM, Dulbecco's modified Eagle's medium; 5-HT, 5-hydroxytryptamine ('serotonin'); MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; PKC, protein kinase C.

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and streptomycin (Biochrom, Berlin, Germany), fetal calf serum (Gibco, Eggenstein, Germany) and bovine insulin (Sigma, Muenchen, Germany). 5-HT was from Sigma, PMA was from Serva (Heidelberg, Germany), and KN62, KN93, PD98059 and GF109203x were from Calbiochem (Bad Soden, Germany). Stock solutions of these compounds were prepared in DMSO. Appropriate controls with the vehicle DMSO were included in all experiments. PVDF membrane was obtained from Pall, Bio-support Division (Dreieich, Germany). The CaM kinase II assay kit was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). The antibody directed against CaM kinase II δ subunit was kindly provided by M. Osterhoff and A. Pfeiffer (Bochum, Germany) [20].

Cell culture

Rat mesangial cells were isolated and cultured as described previously [21]. Cells were grown in DMEM supplemented with 2 mM L-glutamine, 5 μ g/ml insulin, 4.5 g/l glucose, 100 i.u./ml penicillin and 100 μ g/ml streptomycin containing 10% (v/v) fetal calf serum. Mesangial cells were used between passages 12 and 20.

For the experiments, renal mesangial cells (5×10^5 cells/10 ml) were plated in Petri dishes 10 cm in diameter in medium with 10% (v/v) fetal calf serum. At subconfluence (after 3–5 days), cells were growth-arrested by serum deprivation in DMEM containing 0.5% (v/v) fetal calf serum for 3–4 days. Growth-arrested mesangial cells were used in all experiments.

Northern blot analysis

After stimulation for the times indicated, cells were scraped with a rubber 'policeman' into chilled 50 ml polypropylene centrifuge tubes and spun down (225 g, 4 °C). The pellet was lysed by adding 300 μ l of lysis solution containing guanidinium isothiocyanate. Total RNA was extracted in accordance with the protocol of Chomczynski and Sacchi [22], with minor alterations. The RNA yield was usually 30–40 μ g per Petri dish.

Separation of total RNA (10 μ g per lane) was achieved by the use of 1.2% (w/v) agarose gels containing 1.9% (v/v) formaldehyde with 20 mM Mops (pH 7.0)/5 mM sodium acetate/1 mM EDTA as gel/running buffer. Separated RNA was transferred to nylon membranes by capillary blotting and fixed by being baked at 80 °C for 2 h.

Hybridization was performed with cDNA probes labelled with [32 P]dCTP by using the Nonaprimer Labelling Kit II (Appligene) as described [23]. The specific Cox-1 and Cox-2 probes were 2.767 kb and 1.156 kb *Eco*RI fragments from the 5' end of mouse cDNA respectively [24]. The glyceraldehyde-3-phosphate dehydrogenase probe was obtained with a 500 bp reverse-transcribed fragment [7]. Hybridization was performed by standard procedures [6]. DNA–RNA hybrids were detected by autoradiography with Kodak X-OMAT AR film.

Quantitative analysis was performed by densitometric scanning of the autoradiographs (Froebel, Wasserburg, Germany). All values were corrected for differences in RNA loading by calculating the ratio of Cox-2 to the expression of glyceraldehyde-3-phosphate dehydrogenase or 18 S rRNA.

Western blot analysis

Cellular proteins were isolated by using RIPA buffer [50 mM Tris/HCl (pH 7.5)/1% (v/v) Triton X100/1% (w/v) sodium deoxycholate/0.1% SDS/150 mM NaCl/1 mM PMSF/1 mM

sodium vanadate/10 mM sodium fluoride/3 μ g/ml aprotinin]. For Western blot analysis, 10–30 μ g of protein was separated by SDS/PAGE [10% (w/v) gel], transferred to PVDF membrane and probed with specific polyclonal antibodies. Polyclonal rabbit antibodies directed against specific peptides of p44 MAPK or its phosphorylated form, P-p44 MAPK, were obtained from New England Biolabs. Both antibodies detected the 42 and 44 kDa MAPK isoforms. The polyclonal rabbit antibody directed against mouse Cox-1 was kindly provided by D. DeWitt (East Lansing, MI, U.S.A.). Cox-1 expression is not subject to change during brief incubations with the stimuli used and thus was taken as the control for equal protein loading. The polyclonal antibody directed against the C-terminal association domain of CaM kinase II δ_2 was kindly provided by M. Osterhoff and A. Pfeiffer. The peroxidase-conjugated anti-rabbit secondary antibody was obtained from Amersham (Braunschweig, Germany). Protein–antibody complexes were detected by the enhanced chemiluminescence detection system (ECL[®], Amersham).

Determination of p42/p44 MAPK activity

MAPK activity was determined after immunoprecipitation of the MAPKs with myelin basic protein (MBP) as substrate. Cell lysates (approx. 300 μ g of protein) were precleared by incubation with Protein G+-agarose for 1 h at 4 °C. The precleared supernatants were further incubated overnight with 0.2 μ g of specific anti-(p42/p44) antibody (see above). Immune complexes were precipitated by incubation with Protein G+-agarose for 1–3 h. Thereafter, the immune complexes were collected by centrifugation, washed twice with RIPA buffer, twice with kinase buffer [10 mM Hepes (pH 7.4)/10 mM glycerol phosphate/10 mM MgCl₂/1 mM dithiothreitol/0.1 mM sodium vanadate] and then resuspended in 15 μ l of the same buffer. MBP and [γ - 32 P]ATP (5 μ Ci) were added to a final volume of 20 μ l. The enzyme reaction was initiated by incubation at 30 °C and continued for 20 min. The reaction was terminated by rapid cooling on ice, centrifugation for 1 s and mixing of the supernatant with 5 μ l of sample buffer. Proteins were separated by SDS/PAGE [15% (w/v) gel]. After the gel had been dried, the phosphorylated MBP was detected by autoradiography.

Determination of CaM kinase activity

CaM kinase activity was determined in RIPA lysates supplemented with 2 mM EGTA by using an assay kit obtained from Upstate Biotechnology. Protein (2 μ g) was incubated with a specific substrate and inhibitors of other kinases in accordance with the manufacturer's instructions. Ca²⁺/calmodulin-independent activity was determined in the presence of 5 mM EGTA and calculated as a percentage of total activity.

RESULTS

CaM kinases are involved in 5-HT-mediated, but not PMA-mediated, Cox-2 mRNA induction

Incubation of mesangial cells with 5-HT leads to a rapid induction of Cox-2 mRNA expression that is completely abrogated when Ca²⁺ is omitted from the extracellular medium [8]. We investigated the CaM kinase II as one potential target enzyme sensitive to changes in intracellular Ca²⁺ levels. For this purpose we used specific inhibitors, KN62 and KN93, which have been reported not to affect the activities of PKC or protein kinase A even at concentrations 10-fold those used in this study, i.e. 10 and 1 μ M [13,25,26]. Preincubation of the cells with KN62 for 30 min inhibited 5-HT-induced Cox-2 mRNA expression in a

Figure 1 Differential effect of CaM kinase inhibition on 5-HT-mediated and PMA-mediated Cox-2 mRNA expression

Mesangial cells were preincubated with KN62 (10 or 1 μ M) for 30 min as indicated, followed by stimulation with 5-HT (1 μ M) or PMA (TPA, 0.1 μ M) for 2 h. RNA was isolated as described in the Materials and methods section. Expression of specific RNA species was detected by Northern blot analysis. Co, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 1 Interference of CaM kinase inhibitors with the expression of Cox-2 mRNA

Mesangial cells were treated as described in Figure 1. Northern blots were quantified by densitometry. Values obtained for Cox-2 mRNA expression were corrected for differences in RNA loading by calculating the ratio of the expression of Cox-2 to that of glyceraldehyde-3-phosphate dehydrogenase. To compare different experiments, the expression of Cox-2 mRNA obtained by stimulation with 5-HT or PMA was set to 100%. Results are means $\pm$ S.D. and means $\pm$ half range respectively for n independent experiments (given in parentheses). Abbreviation: n.d., not done.

Stimulant	Inhibitor ...	Expression of Cox-2 mRNA (% of control)			
		None (control)	KN62 (10 μ M)	KN62 (1 μ M)	KN93 (10 μ M)
5-HT (1 μ M)		100	35 $\pm$ 9 (5)	50 $\pm$ 11 (6)	38 $\pm$ 12 (2)
PMA (100 nM)		100	103 $\pm$ 8 (3)	97 $\pm$ 10 (4)	n.d.

dose-dependent manner (Figure 1, Table 1). Similar results were also obtained with a second inhibitor of CaM kinases, KN93 (Table 1). The induction of Cox-2 mRNA by PMA was not significantly decreased by either inhibitor. The expression of Cox-1 mRNA was not affected by either treatment.

To characterize CaM kinase II in mesangial cells further, the protein was detected by Western blot analysis with an antibody directed against the C-terminal association domain of CaM kinase II δ_2 [20]. The antibody detected two bands with molecular masses of 55 and 57 kDa (Figure 2). Preincubation of the antibody with the peptide antigen completely abolished the detection of the two proteins. The 55 kDa band co-migrated with the 55 kDa CaM kinase II δ_2 detected in the homogenates of insulinoma cells ([20], and results not shown). CaM kinase activity was determined in cell homogenates after stimulation with 5-HT. A rapid and transient increase in Ca^{2+} /calmodulin-independent activity was observed (Figure 2), the time course of which paralleled the increase in intracellular Ca^{2+} levels detected earlier [10].

p42/44 MAPK is involved in signalling of 5-HT and PMA in mesangial cells

In contrast with the differential effects of the CaM kinase inhibitors, PD98059, an inhibitor of p42/44 MAPK activation

Figure 2 CaM kinase II δ_2 is detected in mesangial cells

To determine CaM kinase activity, mesangial cells were incubated with 5-HT (1 μ M) for the durations indicated. Ca^{2+} /calmodulin-independent activity was determined in the presence of 5 mM EGTA and is expressed as a percentage of total CaM kinase activity. Results are means $\pm$ half range for two independent experiments. Cell homogenate (15 μ g) was subjected to Western blot analysis as described in the Materials and methods section. As a control, the antibody was preincubated with the peptide antigen (+ Ag) before use.

(IC₅₀ 2–7 μ M [27–29]), inhibited the induction of Cox-2 expression by 5-HT as well as by PMA [8]. Activation of MAPK by 5-HT was determined with MBP as substrate (Figure 3, left panel). When the cells were preincubated with PD98059 to inhibit the activation of MAPK, the activity of the MAPK determined after immunoprecipitation was decreased to background levels. The same samples were also analysed by Western blot analysis with the use of antibodies specific for the phosphorylated form of p42/44 MAPK (Figure 3, right panel). As a control for equal loading and blotting of the gel, the 72 kDa protein Cox-1 was detected on the upper part of the blot. Given the coincidence of the MAPK activity assay and the Western blot analysis of MAPK phosphorylation, detection of the phosphorylated form of MAPK was used in all further experiments as a measure of MAPK activation.

In mesangial cells p44 MAPK (ERK1) seemed to be the more abundant isoform, the band of p44 MAPK being more prominent than that of p42 MAPK in all experiments (Figure 4 inset, lower panel). Activation of the cells by 5-HT, however, preferentially increased the phosphorylation and thus the activation of p42 MAPK (ERK2). Activation was rapid, with a maximum after 5 min (Figure 4). Activation of MAPK by 5-HT was insensitive

Figure 3 Correlation of MAPK activity and phosphorylation

Mesangial cells were preincubated with PD98059 (PD, 10 μ M) for 30 min, followed by stimulation with 5-HT (1 μ M) for 5 min. MAPK activity was determined with MBP as substrate (left panel) as described in the Materials and methods section. Protein lysates were also used for Western blot analysis (right panel). On the upper part of the blot Cox-1 (72 kDa) was detected; on the lower part, the phosphorylated forms of p42 and p44 MAPK (P-p42 and P-p44 respectively) were detected with specific antibodies. Co, control.

Figure 4 Kinetics of MAPK phosphorylation after 5-HT stimulation

Mesangial cells were incubated with 5-HT ($1 \mu\text{M}$) for the durations indicated. Phosphorylated (P-p44, P-p42) and non-phosphorylated p42/44 MAPK were detected by Western blot analysis (inset). To compare different experiments, the expression of P-p42 after 10 min of stimulation with 5-HT was set to 100%. Results shown are means $\pm$ S.D. for three experiments.

Figure 5 Activation of MAPK by 5-HT is PKC-dependent

Mesangial cells were preincubated with (+) or without (—) the PKC inhibitor GF109203x ($2 \mu\text{M}$) for 30 min and then stimulated for 5 min with 5-HT ($1 \mu\text{M}$) or PMA (TPA, 0.1 or $0.01 \mu\text{M}$) as indicated. The Western blot was detected with antibodies specific for the phosphorylated form of p42/44 MAPK (P-p42 and P-p44 respectively) and Cox-1. The blot is representative of three independent experiments. Co, control.

to pertussis toxin (results not shown), indicating coupling of the 5-HT receptor to a G-protein of the G_q family.

Activation of MAPK by 5-HT is PKC-dependent

The activation of mesangial cell PKC by PMA led to a concentration-dependent phosphorylation of MAPK (Figure 5). As with activation by 5-HT, p42 MAPK was preferentially phosphorylated. Phosphorylation of MAPK was abolished when the cells were pretreated with the PKC inhibitor GF109203x before stimulation with PMA (Figure 5). Phosphorylation of MAPK by 5-HT was almost completely abolished when PKC was inhibited by GF109203x, indicating PKC as the signalling molecule located between the 5-HT receptor and MAPK.

Activation of CaM kinase is not related to the activation of MAPK

Pretreatment of mesangial cells with the inhibitors of CaM kinase KN62 or KN93 did not interfere with the activation of MAPK by either 5-HT or PMA (Figure 6), excluding CaM kinase as a signalling module upstream of p42/p44 MAPK.

Figure 6 CaM kinase activity is not essential for 5-HT- or PMA-induced MAPK activity

Mesangial cells were preincubated with KN62 or KN93 ($10 \mu\text{M}$) for 30 min and then stimulated with 5-HT ($1 \mu\text{M}$) or PMA (TPA, $0.1 \mu\text{M}$) for 5 min. The Western blot was detected with antibodies specific for the phosphorylated form of p42/44 MAPK (P-p42 and P-p44 respectively) and Cox-1. The expression of Cox-1 served as a control for equal loading and blotting. The blot is representative of three independent experiments.

Figure 7 Additive effects of PKC and CaM kinase inhibition on the 5-HT-stimulated expression of Cox-2 mRNA

Mesangial cells were preincubated with PMA (T, $0.1 \mu\text{M}$) overnight and/or preincubated with KN62 for the last 30 min (K, $1 \mu\text{M}$). The cells were then left untreated (Co) or stimulated with 5-HT ($1 \mu\text{M}$) for 2 h.

Table 2 Decrease in 5-HT-stimulated expression of Cox-2 mRNA by inhibition of PKC and CaM kinase

Mesangial cells were preincubated overnight in the presence or absence of 100 nM PMA and then treated with or without $10 \mu\text{M}$ KN62 for 30 min before stimulation with 5-HT for 2 h. Preincubations were also performed with control cells. Values obtained for Cox-2 mRNA expression were corrected for differences in RNA loading by calculating the ratio of expression of Cox-2 mRNA to that of 28 S rRNA. To compare different experiments, the expression of Cox-2 mRNA obtained by stimulation with 5-HT was set to 100%. Results are means $\pm$ S.D. for three independent experiments. * $P < 0.02$ as compared with incubation without KN62 (Student's t test).

[5-HT] (μM)	Expression of Cox-2 mRNA (% of that of 5-HT-stimulated cells)			
	—PMA/—KN62	—PMA/+KN62	+PMA/—KN62	+PMA/+KN62
0 (control)	29.6 $\pm$ 4.2	17.1 $\pm$ 7.3	31.0 $\pm$ 4.3	15.9 $\pm$ 7.7*
1	100	65.2 $\pm$ 11.5	55.0 $\pm$ 10.9	19.4 $\pm$ 8.4*

When PKC was down-regulated by prolonged incubation with PMA, the remaining 5-HT-mediated Cox-2 mRNA expression was completely suppressed when the cells were preincubated with PMA plus KN62 (Figure 7 and Table 2), suggesting the parallel activation of PKC and CaM kinase rather than successive

activation. Unstimulated mesangial cells showed a basal, low expression of Cox-2 mRNA (Figures 1 and 7). Incubation with KN62, but not down-regulation of PKC, decreased basal levels of Cox-2 mRNA expression.

DISCUSSION

The various 5-HT receptors couple to different intracellular signalling pathways via distinct G-proteins. In mesangial cells the induction of early response genes and increased proliferation are mediated via pertussis-toxin-insensitive 5-HT_{2A} receptors that couple to G-proteins of the G_q family [8,30]. In connection with further downstream signalling pathways, we have shown previously that Ca²⁺, PKC and MAPK are essential for the induction of Cox-2 mRNA expression, whereas the phosphatidylinositol-specific phospholipase C is not critically involved and the activation of protein kinase A interferes negatively with gene expression [8]. In the present study we showed that the activation of p42/44 MAPK by 5-HT is downstream of PKC, whereas the activation of CaM kinase represents an independent pathway.

Activation of the MAPK pathway has been observed after stimulation of various types of receptor (reviewed in [31,32]). In mesangial cells, activation of MAPK by 5-HT was dependent on PKC activation. The inhibitor used, GF109203x, is a potent and selective inhibitor of PKC [33] and preferentially inhibits the diacylglycerol-activated types of PKC [34]. Consistently, the down-regulation of these PKCs by prolonged incubation with PMA also prevented the 5-HT-mediated induction of Cox-2 mRNA expression. The PKC-associated activation of MAPK has been reported for other G_q-coupled receptors, e.g. endothelin or vasopressin receptors [31]. However, the direct substrate downstream of PKC has not yet been defined, although there is evidence that the kinase c-Raf rather than the small G-protein Ras is involved in this pathway [31,35].

Incubation of mesangial cells with 5-HT leads to an increase in intracellular [Ca²⁺] by the release of Ca²⁺ from internal stores as well as the influx of extracellular Ca²⁺ by voltage-insensitive Ca²⁺ channels ([10], and references therein). Ca²⁺ proved to be essential for the induction of Cox-2 mRNA expression not only by 5-HT but also by stimuli acting via tyrosine kinase receptors such as platelet-derived growth factor [12]. Inhibition of the CaM phosphatase calcineurin by cyclosporin A decreased Cox-2 mRNA expression by approx. 50 %, thus including this Ca²⁺-sensitive enzyme in the signalling pathway leading to Cox-2 mRNA expression [7,36]. By the use of specific inhibitors, CaM kinases, most probably CaM kinase II (see below), were now also shown to be regulatory elements of Cox-2 mRNA expression.

The inhibitors of CaM kinases used, KN62 and KN93, inhibit different types of CaM kinase [13,37]. In respect of signal transduction, it was shown by assays *in vitro* that CaM kinases II and IV were able to phosphorylate the cAMP-response element-binding protein (CREB), but only CaM kinase IV enhanced transcription through the cAMP-response element (CRE)/CREB system [37]. In mesangial cells, elevation of cAMP levels and activation of protein kinase A inhibited rather than induced Cox-2 expression [8]. Our results thus indicate that the CRE/CREB system is not involved in the 5-HT-mediated induction of Cox-2 mRNA, suggesting that CaM kinase II is the most likely target of KN62 and KN93 in the signalling pathway investigated. This was confirmed by the detection of CaM kinase II δ_2 by Western blot analysis. The antibody detected two bands, one of which co-migrated at 55 kDa with the enzyme identified in insulinoma cells [20]. The second band was also described in RINm5F cells [38] and was specifically recognized by the antibody as shown by preincubation with the peptide antigen. Its identity

remains to be determined. CaM kinase II δ_2 thus seems to be the major isoform expressed in mesangial cells. These results are in accordance with the expression of CaM kinase δ isoforms in other peripheral cells, not only insulinoma cells, but also the more closely related vascular smooth-muscle cells of rat aorta [39].

Activation of CaM kinase II is characterized by auto-phosphorylation and subsequent Ca²⁺/calmodulin-independent phosphorylation of specific substrates [18]. This autonomous CaM kinase II activity was detected in vascular smooth-muscle cells after stimulation with various agonists, all of which increased intracellular Ca²⁺ levels [18]. In agreement with the results reported by these authors, a transient increase in autonomous CaM kinase activity was observed in mesangial cells stimulated with 5-HT, which paralleled the increase in intracellular [Ca²⁺] characterized previously [10].

The downstream target of CaM kinase II in mesangial cells is not yet known. Possible candidates involve the transcription factor C/EBP β , which is phosphorylated by CaM kinase II [40] and has a potential binding site within the Cox-2 promoter [41].

The activation of CaM kinase seems to have a modulatory role in Cox-2 induction because even concentrations of KN93 and KN62, which have been reported to inhibit CaM kinase II activation completely [25,26], inhibited Cox-2 induction only partly, whereas the inhibition of PKC or MAPK led to a much more pronounced decrease in Cox-2 mRNA expression. The partial inhibition of Cox-2 expression by CaM kinase inhibitors correlates with the partial inhibition observed when mesangial cells are treated with cyclosporin A to inhibit calcineurin phosphatase ([7]; J. Heusinger-Ribeiro and M. Goppelt-Struebe, unpublished work).

In cultured mesangial cells Cox-2 shows a low but distinct basal mRNA and protein expression that might relate to the fact that, even when they are serum-starved, these cells cannot be completely growth-arrested. The basal expression of Cox-2 as a dynamic state becomes obvious when the cells are treated with cycloheximide: the Cox-2 mRNA levels increase, most probably owing to the inhibition of newly synthesized degrading enzymes [7]. The basal expression of Cox-2 was decreased significantly by incubation of the cells with the inhibitors of CaM kinase II, suggesting a role for this enzyme in maintenance of the basal turnover of Cox-2 mRNA.

Taken together, our results indicate that in mesangial cells, which are non-transformed, primary cell cultures, the PKC–MAPK pathway has a major role in the 5-HT-mediated expression of Cox-2. It is supplemented by the CaM kinase pathway, which is also involved in the basal expression of Cox-2 mRNA. In additional studies the downstream elements need to be defined that contribute, with other signalling elements, to the fine-tuning of Cox-2 mRNA expression-stimulated cells.

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