British Journal of Pharmacology (2006) 148, 833–844
www.nature.com/bjp

Profiling of functional phosphodiesterase in mesangial cells using a CRE-SEAP-based reporting system

[1,2,3]Ying Zhu, [1]Jian Yao, [1]Yiman Meng, [1]Ayumi Kasai, [1]Nobuhiko Hiramatsu, [1]Kunihiro Hayakawa, [3]Takashi Miida, [2]Masayuki Takeda, [3]Masahiko Okada & *,[1]Masanori Kitamura

[1]Department of Molecular Signaling, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Chuo, Yamanashi 409-3898, Japan; [2]Department of Urology, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi, Japan and [3]Department of Preventive Medicine, Graduate School of Medicine and Dental Science, University of Niigata, Niigata, Japan

1 Phosphodiesterases (PDEs) are critically implicated in the regulation of mesangial cell function, but profile of functional PDEs in mesangial cells is still unclear. In this study, we investigated roles of individual PDEs in the regulation of mesangial cell behavior by the cAMP pathway.

2 Reporter mesangial cells that express secreted alkaline phosphatase (SEAP) under the control of the cAMP response element (CRE) were exposed to selective PDE inhibitors in the presence or absence of cAMP, and activity of CRE, expression of CRE-regulated protein, mitogenesis and cell survival were examined.

3 Exposure of reporter cells to cAMP-elevating agents resulted in time- and concentration-dependent activation of CRE. Treatment of the cells with any PDE inhibitors alone did not induce CRE activation. Under stimulation with 8-bromo-cAMP or 8-bromo-cGMP, however, inhibitors of PDE2, PDE3, PDE4 and PDE5 enhanced activation of CRE. Inhibition of PDE1 or PDE6 did not affect the CRE activation.

4 Among different combinations tested, only inhibitors of PDE3 and PDE4 cooperatively increased the level of intracellular cAMP, activity of protein kinase A, activation of CRE, and CRE-regulated protein, connexin43.

5 Concomitant inhibition of PDE3 and PDE4 attenuated mitogen-induced activation of extracellular signal-regulated kinases and cell proliferation. Under serum deprivation, combinational inhibition of PDE3 and PDE4 exclusively caused activation of caspase-3 and apoptosis.

6 The present data elucidated that PDE3 and PDE4 play critical roles in the regulation of mesangial cell function. PDE3 and PDE4 were identified as the novel, antiapoptotic machinery that supports survival of mesangial cells.
British Journal of Pharmacology (2006) **148,** 833–844. doi:10.1038/sj.bjp.0706785;
published online 5 June 2006

Keywords: Mesangial cell; phosphodiesterase (PDE); cAMP; apoptosis; secreted alkaline phosphatase (SEAP)

Abbreviations: 8-Br-cAMP, 8-bromo-cAMP; 8-Br-cGMP, 8-bromo-cGMP; CRE, cAMP response element; Cx43, connexin43; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; FSK, forskolin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1β, interleukin-β; LDH, lactate dehydrogenase; MAP kinase, mitogen-activated protein kinase; 8-pCPT-cGMP, 8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate; PDE, phosphodiesterase; PDGF, platelet-derived growth factor; PKA, protein kinase A; SEAP, secreted alkaline phosphatase; SNAP, *S*-nitroso-*N*-acetylpenicillamine; TNF-α, tumor necrosis factor-α; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; VASP, vasodilator-stimulated phosphoprotein

Introduction

Cyclic adenosine 3′,5′ monophosphate (cAMP) is an important second messenger that mediates various cellular responses to external stimuli. cAMP is synthesized by a family of cyclases and degraded by phosphodiesterases (PDEs). The family of PDE consists of at least 11 isozymes with different substrate specificity, subcellular kinetics and sensitivity to effector molecules (Beavo, 1995; Houslay & Milligan, 1997). PDEs are expressed in various organs in tissue- and cell type-specific manners, allowing PDEs as possible therapeutic targets for the treatment of local diseases (Dousa, 1999). In the kidney, mesangial cells express several PDEs including PDE1, PDE3, PDE4 and PDE5 (Matousovic *et al*., 1995; Dousa, 1999). Previous reports showed that inhibition of some PDEs suppressed proliferation, migration and production of reactive oxygen metabolites in cultured mesangial cells (Chini *et al*., 1994; 1997a, b; Matousovic *et al*., 1995; Dousa, 1998; 1999). Tsuboi *et al*. (1996) demonstrated that *in vivo* administration of PDE3 and PDE4 inhibitors prevented development of mesangial proliferative glomerulonephritis in rats. Therapeutic utility of PDE4 inhibitors was also reported in experimental crescentic glomerulonephritis and endotoxin-induced acute renal failure (Begany *et al*., 1996; Tam *et al*., 2000). Currently, however, pathophysiological roles of individual PDEs in the kidney have not been fully elucidated.

*Author for correspondence; E-mail: masanori@yamanashi.ac.jp

Activity of cellular PDE is usually evaluated by the ability of cellular extracts to hydrolyze cAMP or by the increase in intracellular cAMP after exposure to PDE inhibitors (Matousovic *et al*., 1995; Dousa, 1998; 1999). However, alteration in the total cellular cAMP may not always reflect the ability of PDEs to activate the protein kinase A (PKA)-mediated signaling events. PDE inhibitors may cause accumulation of cAMP in the local compartment without detectable alterations in total cAMP (Matousovic *et al*., 1995; Dousa, 1998; 1999). It has been suggested that evaluation of PKA activity or activity of downstream molecules may be more sensitive indicators than measurement of total cAMP content (Dufau *et al*., 1977; Chini *et al*., 1994).

Activation of PKA is followed by phosphorylation of cAMP response element (CRE)-binding proteins and subsequent activation of CRE, leading to expression of CRE-regulated genes. In the present report, we established and used a CRE-based reporter system to evaluate roles of individual PDEs in the function of mesangial cells. In this approach, mesangial cells were engineered to express secreted alkaline phosphatase (SEAP) under the control of CRE.

The SEAP reporter system (Berger *et al*., 1988) has been used for many years to investigate activity of known or putative enhancer/promoter elements. Normally, alkaline phosphatase is not secreted, but the recombinant SEAP originated from placental alkaline phosphatase is efficiently secreted from transfected cells. In SEAP-transfected cells, the level of SEAP activity detected in culture media is directly proportional to changes in SEAP mRNA and protein (Berger *et al*., 1988; Cullen & Malim, 1992). This property allows SEAP to serve as a quantitative reporter for gene expression. As a reporter molecule, SEAP has several advantages over other reporters including luciferase, green fluorescent protein and β-galactosidase. Because preparation of cell lysates is not required, it is possible to monitor the activity of certain promoters/enhancers continuously using identical cell cultures. The assay of SEAP is faster, easier and more sensitive than assays using other reporter enzymes (Kasai *et al*., 2004; 2005). Furthermore, in contrast to secreted luciferase, the SEAP reporter system is also useful for *in vivo* monitoring of certain regulatory elements (Hiramatsu *et al*., 2005; Meng *et al*., 2005b).

In the present study, we investigated roles of PDEs in the control of mesangial cell behavior using the CRE–SEAP-based reporter system. Our data elucidated that, among various PDEs, PDE3 and PDE4 play critical roles in the regulation of mesangial cell function. Our data also disclosed that PDE3 and PDE4 function as the novel, antiapoptotic machinery that supports survival of mesangial cells.

Methods

Reagents

PDE inhibitors used in this study were; vinpocetine [PDE1 inhibitor (IPDE1); 50 μM], erythro-9-(2-hydroxy-3-nonyl) adenine [PDE2 inhibitor (IPDE2); 100 μM], cilostamide [PDE3 inhibitor (IPDE3); 20 μM], rolipram [PDE4 inhibitor (IPDE4); 20 μM], zaprinast [PDE5 inhibitor (IPDE5); 100 μM] and dipyridamole [PDE6 inhibitor (IPDE6); 100 μM]. Cilostamide was purchased from Wako (Osaka, Japan), and others were from Sigma (St Louis, MO, U.S.A.). Human platelet-derived growth factor (PDGF)-BB was obtained from Perro Tech Inc. (Rocky Hill, NJ, U.S.A.). Human recombinant interleukin-1β (IL-1β) and human recombinant tumor necrosis factor-α (TNF-α) were generous gifts of Otsuka Pharmaceutical Co. Ltd (Tokushima, Japan) and Dr Katsuo Noguchi (Teikyo University School of Medicine, Tokyo, Japan), respectively. All other reagents were purchased from Sigma.

Establishment of reporter cells

Clonal mesangial cells (SM43) were established from isolated renal glomeruli of a male Sprague–Dawley rat and identified as being of the mesangial cell phenotype as described before (Kitamura *et al*., 1994). Cells were maintained in Dulbecco's modified Eagle's medium/F-12 (Gibco-BRL, Gaithersburg, MD, U.S.A.) supplemented with 100 U ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin, 0.25 μg ml^{-1} amphotericin B and 5% fetal bovine serum (FBS). Using a calcium-phosphate co-precipitation method, SM/CRE-SEAP15 cells were established by transfection of SM43 cells with pCRE-SEAP (BD Biosciences, Palo Alto, CA, U.S.A.) together with pcDNA3.1 (Invitrogen, Carlsbad, CA, U.S.A.), as described previously (Meng *et al*., 2005a; Yao *et al*., 2005). pCRE-SEAP encodes SEAP under the control of three copies of CRE. Medium containing 1% FBS was generally used for studies, except for experiments on cell survival in which serum-free medium was used.

Western blot analysis

Extracted cellular proteins were separated by 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels and electrotransferred onto polyvinylidine difluoride membranes. After blocking with 3% bovine serum albumin in phosphate-buffered saline (PBS), the membranes were incubated with anti-vasodilator-stimulated phosphoprotein (VASP) antibody (dilution 1 : 1000; Chemicon International, Temecula, CA, U.S.A.), anti-connexin43 (Cx43) antibody (dilution 1 : 2000; Sigma), anti-caspase-3 antibody (dilution 1 : 1000; Cell Signaling, Beverly, MA, U.S.A.), or anti-phospho-mitogen-activated protein (MAP) kinase antibody (dilution 1 : 1000; Cell Signaling). After washing with PBS containing 0.1% Tween 20, the filters were probed with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG, and the bands were visualized by the enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, U.K.). To confirm equal loading of proteins, the filters were soaked in 62.5 mM Tris-HCl (pH 6.8) containing 2% SDS and 100 mM β-mercaptoethanol for 30 min at 60°C and reprobed with monoclonal anti-β-actin antibody (1 : 30,000 dilution; Sigma) or anti-extracellular signal-regulated kinase (ERK) antibody (dilution 1 : 1000; Cell Signaling). Densitometric analysis was performed using Scion Image (Scion Corporation, Frederick, MO, U.S.A.).

Northern blot analysis

Total RNA was extracted by a single-step method, and Northern blot analysis was performed as described before (Kitamura *et al*., 1996; Yao *et al*., 2005). The SEAP cDNA

(BD Biosciences) was radio-labeled and used as a probe. Expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

SEAP assay

Activity of SEAP was evaluated using the Great EscAPe™ Detection Kit (BD Biosciences) following the protocol provided by the manufacturer. In brief, reporter cells in 96-well plates were exposed to stimuli for 24 h, and culture media were collected and centrifuged at $12{,}000 \times g$ for 2 min. Fifteen microliters of dilution buffer was mixed with 5 μl of the sample, and the mixture was incubated at 65°C for 30 min to eliminate endogenous alkaline phosphatase activity. Twenty microliters of assay buffer was subsequently added to the mixture and incubated for additional 10 min at room temperature. CSPD chemiluminescence substrate was diluted with 20 × chemiluminescence enhancer to 1.25 mM, and 20 μl of the diluted substrate was added to each sample, followed by 10 min incubation at room temperature. The intensity of chemiluminescent signal was determined by a luminometer (Gene Light 55; Microtech Nition, Chiba, Japan). Assays were performed in quadruplicate.

Assessment of cAMP

Confluent mesangial cells in 24-well culture plates were treated with PDE inhibitors for 1 h, and the cells were lysed and assayed for cAMP using cAMP Biotrak Enzymeimmunoassay System (Amershan Biosciences) according to the instructions provided by the manufacturer.

Assessment of cell growth

Mesangial cells in 24-well culture plates were exposed to various PDE inhibitors in the presence or absence of PDGF for 48 h. The cells were washed with PBS and trypsinized. The cell number per well was counted.

Cytotoxicity assay

Cytotoxicity was evaluated by the release of lactate dehydrogenase (LDH). LDH, a stable cytoplasmic enzyme, is released into culture media upon damage of the cell membrane. Confluent mesangial cells in 96-well culture plates were exposed to PDE inhibitors for 24 h, and culture media were collected and assayed for LDH using LDH Cytotoxicity Detection Kit (Takara Bio Inc., Otsu, Shiga, Japan).

Assessment of apoptosis

Mesangial cells seeded in 24-well culture plates were exposed to stimuli for 12 h under a serum-free culture condition. Apoptosis was evaluated by staining with Hoechst33258 (Sigma), as described previously (Ishikawa & Kitamura, 2000; Hiramatsu *et al*., 2004). In brief, cells were fixed in 2% formaldehyde for 10 min, stained by Hoechst33258 (10 μg ml^{-1}) for 20 min and subjected to fluorescence microscopy. Apoptosis was identified using morphological criteria including shrinkage of the cytoplasm and nuclear condensation/fragmentation.

Statistical analysis

Assays were performed in triplicate or quadruplicate, and data were expressed as means ± s.e. Statistical analysis was performed using the one-way analysis of variance and the Dunnett test. Comparison of two populations was performed by Student's *t*-test. $P < 0.01$–0.05 was considered to be a statistically significant difference.

Results

Responses of reporter mesangial cells to cAMP and other stimuli

The reporter mesangial cell SM/CRE-SEAP15 was established by stable transfection of SM43 cells with pCRE-SEAP that introduces a SEAP gene under the control of CRE. Figure 1a shows dose-dependent induction of SEAP mRNA in response to forskolin in the established reporter cells. Modest induction of SEAP was observed at 0.1 μM, and the induction was peaked to maximum at 10–50 μM. Consistent with this result, SEAP assay on culture medium revealed that activity of SEAP was induced by forskolin in a dose-dependent manner (Figure 1b). The level of SEAP increased approximately 50-fold by the treatment with 50 μM forskolin. The induction of SEAP by forskolin was not observed in mock-transfected cells that express *neo* alone (data not shown). Time-lapse experiments revealed that the induction of SEAP was detectable within 4–6 h after the stimulation with 10 μM forskolin (Figure 1c). SM/CRE-SEAP15 cells also responded to other cAMP-elevating agents including cAMP analogue 8-bromo-cAMP (8-Br-cAMP; 2 mM) and nonselective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX; 250 μM) (Figure 1d). The induction of SEAP by 8-Br-cAMP was dose-dependent, and the minimum concentration required was 500 μM (Figure 1e). Similarly, cGMP analogue 8-Br-cGMP that activates the cAMP signaling pathway *via* inhibition of PDE3 and/or direct activation of PKA (Sausbier *et al*., 2000; Osinski *et al*., 2001) significantly induced SEAP activity at a relatively high concentration (2 mM) (Figure 1f). In contrast, none of activators for the nuclear factor-κB pathway (IL-1β and TNF-α), the protein kinase C – activator protein 1 pathway (12-*O*-tetradecanoylphorbol-13-acetate; TPA) and the MAP kinase – ternary complex factor pathway (PDGF) triggered induction of SEAP mRNA (Figure 1g) and SEAP activity (Figure 1h), confirming the selectivity and specificity of the response of the established reporter cells.

Profiling of functional PDEs in mesangial cells

Using the established reporter cells, we examined roles of individual PDEs in the regulation of the cAMP pathway in mesangial cells. The reporter cells were treated with selective inhibitors of PDEs (IPDE1–IPDE6), and activity of SEAP in culture medium was evaluated. Under unstimulated conditions, inhibition of individual PDEs alone did not induce obvious activation of the cAMP pathway (Figure 2a). Only IPDE3 slightly increased the level of SEAP, but the induction was only 1.54-fold. However, under modest activation of the cAMP pathway by 500 μM 8-Br-cAMP (3.72 ± 0.76 fold), IPDE3 dramatically increased the level of SEAP (28.36 ± 4.54

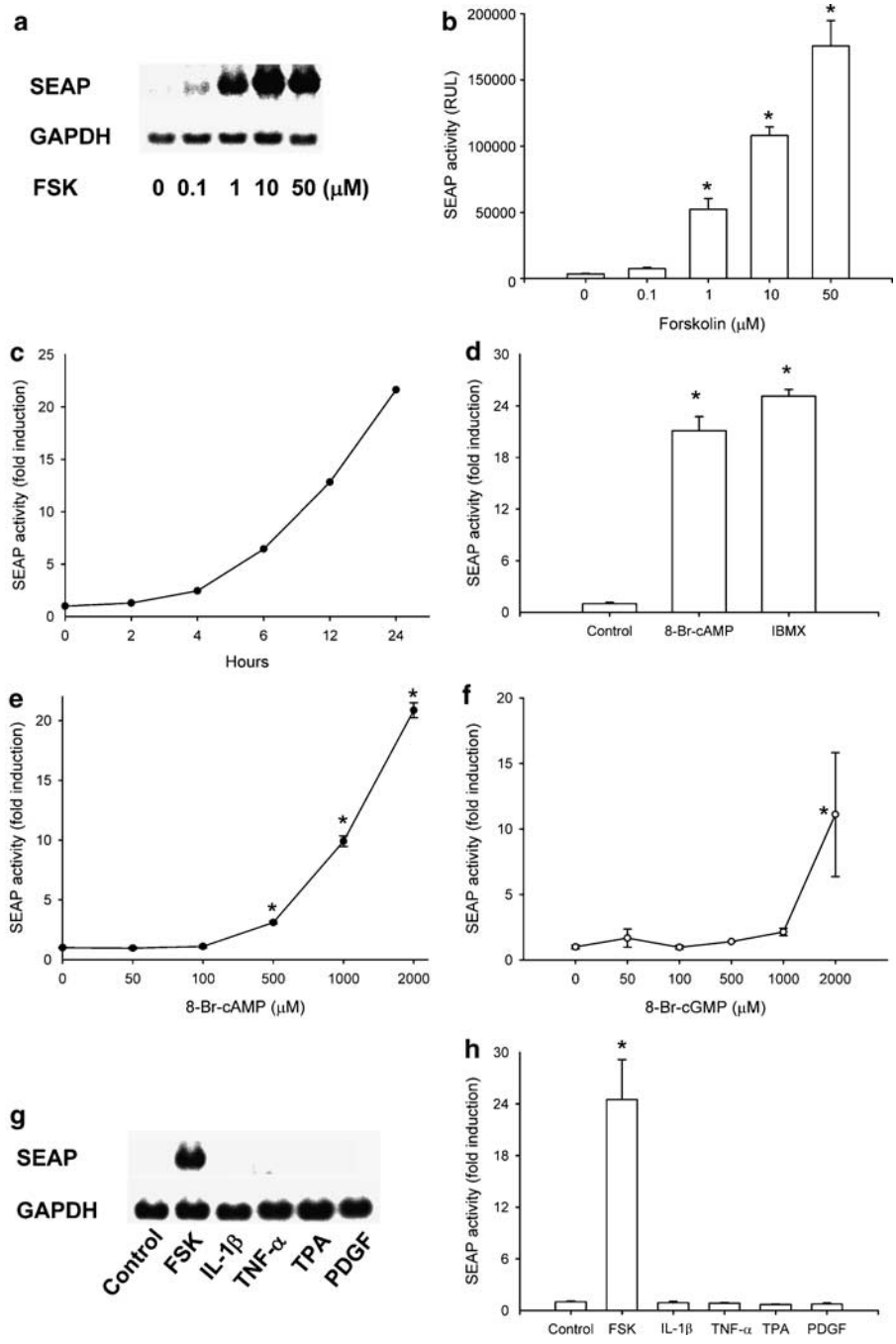

Figure 1 Responses of reporter mesangial cells to cAMP and other stimuli. SM/CRE-SEAP15 reporter cells were established by transfection of SM43 mesangial cells with pCRE-SEAP that introduces SEAP under the control of the CRE. (a, b) Dose-dependent induction of SEAP mRNA and SEAP activity by forskolin (FSK). Reporter cells were incubated with 0.1–50 μM forskolin for 12–24 h, and the cells and culture media were subjected to Northern blot analysis (a) and SEAP assay (b), respectively. Expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was shown as a loading control. RLU, relative light unit. (c) Kinetics of SEAP activity. Reporter cells were exposed to 10 μM forskolin for indicated time periods, and culture media were subjected to chemiluminescence assay to evaluate SEAP activity. (d) Induction of SEAP activity by other cAMP-elevating agents 8-bromo-cAMP (8-Br-cAMP) and IBMX. Reporter cells were exposed to 2 mM 8-Br-cAMP or 250 μM IBMX for 24 h, and culture media were subjected to SEAP assay. (e, f) Dose-dependent induction of SEAP activity by 8-Br-cAMP (e) and 8-bromo-cGMP (8-Br-cGMP) (f). (g, h) Influence of cAMP-unrelated stimuli on SEAP mRNA and SEAP activity. Reporter cells were incubated with 10 μM forskolin, 20 ng ml^{-1} interleukin-1β (IL-1β), 250 U ml^{-1} TNF-α, 10^{-6} M TPA, or 20 ng ml^{-1} PDGF for 8–24 h, and the cells and culture media were subjected to Northern blot analysis (g) and SEAP assay (h), respectively. Data were shown as means $\pm$ s.e. Asterisks indicate statistically significant differences ($P<0.01$).

fold; Figure 2b). IPDE2, IPDE4 and IPDE5 also modestly increased the level of SEAP (IPDE2: 7.24 $\pm$ 0.79 fold; IPDE4: 5.64 $\pm$ 1.16 fold; IPDE5: 7.08 $\pm$ 0.72 fold), whereas IPDE1 and IPDE6 were ineffective. Consistently, under the stimulation with 1 mM 8-Br-cGMP, IPDEs enhanced activation of the cAMP pathway markedly by IPDE3, moderately by IPDE4

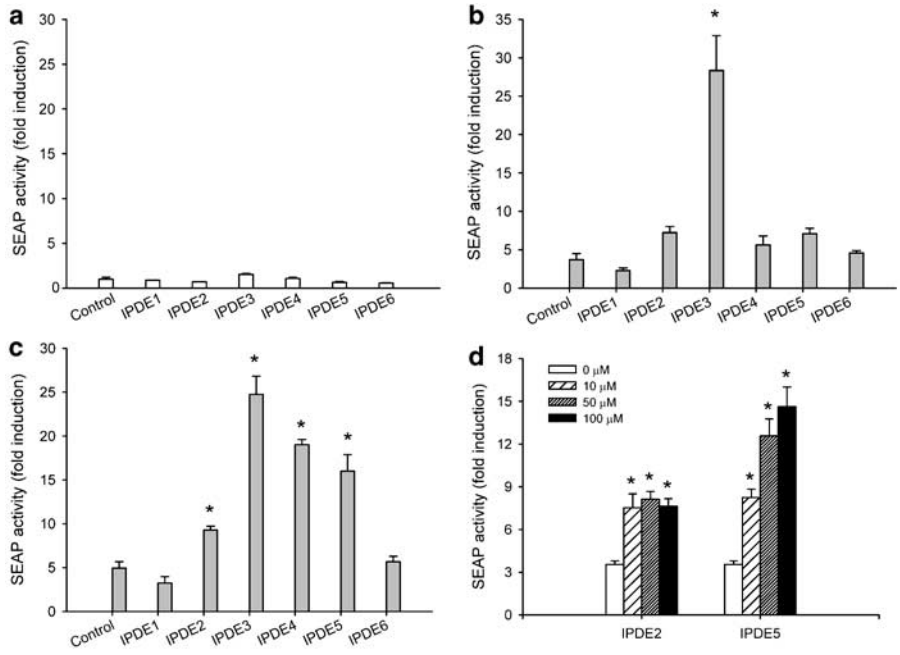

Figure 2 Influence of PDE inhibitors on the activity of the cAMP-CRE pathway. Reporter cells were treated with vinpocetine [PDE1 inhibitor (IPDE1); 50 μM], erythro-9-(2-hydroxy-3-nonyl) adenine [PDE2 inhibitor (IPDE2); 100 μM], cilostamide [PDE3 inhibitor (IPDE3); 20 μM], rolipram [PDE4 inhibitor (IPDE4); 20 μM], zaprinast [PDE5 inhibitor (IPDE5); 100 μM] and dipyridamole [PDE6 inhibitor (IPDE6); 100 μM] for 24 h in the absence (a) or presence of 500 μM 8-Br-cAMP (b) or 1000 μM 8-Br-cGMP (c, d), and activity of SEAP was evaluated by chemiluminescence assay. In (d), dose-dependent effects of IPDE2 and IPDE5 were evaluated. Data were shown as means $\pm$ s.e, and asterisks indicate statistically significant differences ($P<0.01$).

and IPDE5, and modestly by IPDE2 (Figure 2c). Similar to the result shown in Figure 2b, inhibition of PDE1 or PDE6 did not affect the level of SEAP. Of note, neither IPDE1 nor IPDE6 exhibited cytotoxic effects at the concentrations tested, when evaluated by release of LDH (data not shown).

Induction of the cAMP pathway by IPDE2 and IPDE5, the inhibitors of major cGMP-degrading enzymes, was unpredictable. The stimulatory effects could be due to nonselective inhibition of PDEs caused by a relatively high concentration of the inhibitors (100 μM). To exclude this possibility, we examined effects of IPDE2 and IPDE5 at lower concentrations. As shown in Figure 2d, significant activation of CRE by these inhibitors was still observed even at the concentration of 10 μM.

As shown in Figure 2a, inhibition of any single PDE did not substantially activate the cAMP pathway. However, IBMX, a nonselective inhibitor of PDE, markedly increased the level of SEAP in reporter mesangial cells (Figure 1d). We speculated that cooperation of multiple PDEs may suppress the cAMP pathway under the basal culture condition. To examine this possibility, reporter cells were treated with couples of IPDEs. All possible combinations were tested, and we found that concomitant inhibition of PDE3 and PDE4 exclusively activated the cAMP pathway (Figure 3a and b). In the presence of IPDE3, for example, only IPDE4, but not others, induced SEAP activity. The induction by IPDE3 plus IPDE4 was comparable to that by IBMX at the most effective concentration (250 μM) (Figure 3a). Similarly, in the presence of IPDE4, only IPDE3, but not others, induced SEAP activity (Figure 3b). All other combinations tested did not significantly induce activation of the cAMP pathway (data not shown).

To confirm the cooperative roles of PDE3 and PDE4, another PDE3 inhibitor milrinone and PDE4 inhibitor Ro20-1724 were tested. Consistent with the results shown in Figure 3a and b, combinational treatment of reporter cells with these inhibitors led to dramatic activation of CRE, the level of which was comparable to that achieved by IPDE3 (cilostamide) and IPDE4 (rolipram) (Figure 3c). In addition, the cooperative effects of IPDE3 and IPDE4 was not caused by nonspecific effects of these inhibitors used at a relatively high concentration (20 μM), because the significant activation of CRE was still observed even at the low concentration, 1 μM (Figure 3d).

To further confirm the critical, combinational roles of PDE3 and PDE4 in the regulation of the cAMP pathway, levels of intracellular cAMP were evaluated after exposure to PDE inhibitors. As shown in Figure 4a, concomitant inhibition of PDE3 and PDE4 exclusively increased the level of cAMP. Inhibition of PDE3 or PDE4 alone had little influence on the level of cAMP. Concomitant inhibition of PDE2 and PDE5 also did not alter the level of cAMP. Consistent with these results, combinational inhibition of PDE3 and PDE4 exclusively activated cAMP-dependent protein kinase, PKA. That is, phosphorylation of a PKA substrate VASP at serine 157 was markedly induced exclusively by IPDE3 plus IPDE4 (Figure 4b). Inhibition of individual PDE (PDE1–PDE6) alone or PDE2 plus PDE5 did not cause VASP phosphorylation.

To examine whether combinational inhibition of PDE3 and PDE4 indeed altered levels of molecules downstream of the cAMP pathway, we examined the level of cAMP-inducible gap junction protein Cx43 (Chanson *et al*., 1996; TenBroek *et al*., 2001; Bailey *et al*., 2002). Western blot analysis showed that treatment with IPDE3 or IPDE4 alone did not affect the basal level of Cx43. However, concomitant inhibition of PDE3 and

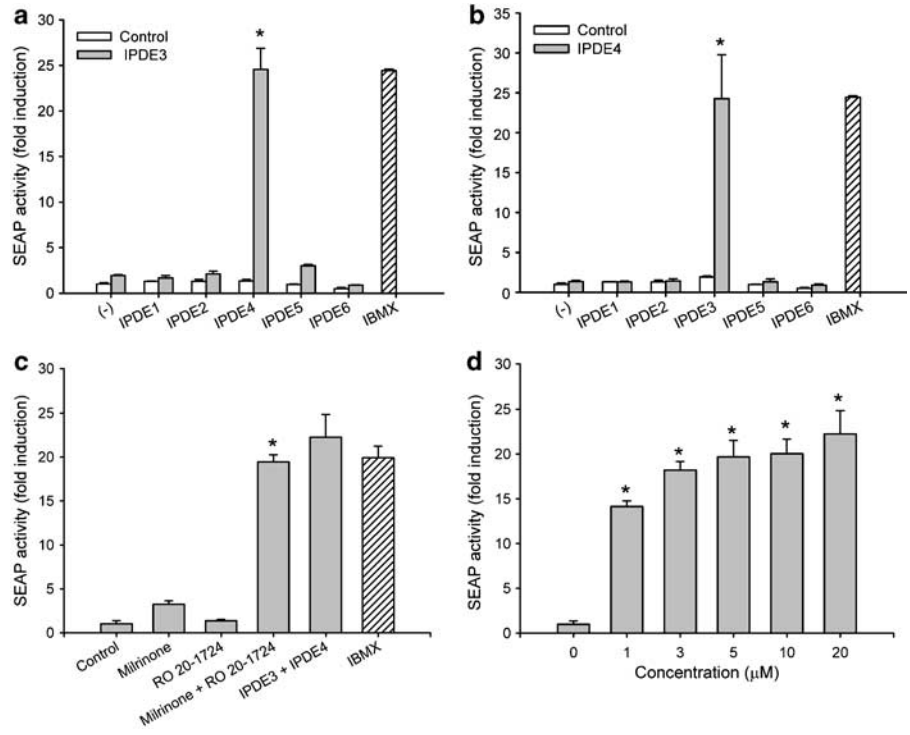

Figure 3 Activation of CRE by inhibition of PDE3 and PDE4. (a, b) Effects of combinational inhibition of PDEs on the activity of CRE. Reporter cells were treated with IPDE1, IPDE2, IPDE3, IPDE4, IPDE5 or IPDE6 in the absence (Control) or presence of IPDE3 (a) or IPDE4 (b) for 24 h. The culture media were subjected to SEAP assay. Treatment with nonselective PDE inhibitor IBMX (250 μM) was used as a positive control. (c) Induction of CRE activation by different inhibitors of PDE3 and PDE4. Reporter cells were treated with milrinone (PDE3 inhibitor; 20 μM), Ro20-1724 (PDE4 inhibitor; 20 μM), IBMX, milrinone plus Ro20-1724 or cilostamide (IPDE3) plus rolipram (IPDE4) for 24 h, and the culture media were subjected to SEAP assay. (d) Dose-dependent activation of CRE by concomitant inhibition of PDE3 and PDE4. Reported cells were exposed to the indicated concentrations of IPDE3 and IPDE4. Data were shown as means $\pm$ s.e, and asterisks indicate statistically significant differences ($P<0.01$).

PDE4 substantially increased the level of Cx43 protein (Figure 5a and b). The similar effect was observed by IBMX, but not by IPDE2 plus IPDE5.

Regulation of mitogenesis and apoptosis of mesangial cells by PDE3 and PDE4

Cx43 is involved in the control of cell growth and survival (Goodenough *et al*., 1996; Kumar & Gilula, 1996). PDE3 and PDE4 possibly regulate mitogenic responses and apoptosis of mesangial cells. To examine this possibility, we first evaluated effects of IPDE3 and IPDE4 on the mitogenic pathway. PDGF is a well-known mitogen that triggers the MAP kinase pathway in mesangial cells (Shulz *et al*., 1988). As shown in Figure 6a and b, treatment of mesangial cells with PDGF markedly induced phosphorylation of ERK1 and ERK2. Treatment of the cells with IPDE3 plus IPDE4 attenuated the PDGF-induced activation of ERKs, which was similar to the effect of IBMX. To a lesser extent, IPDE3 alone also inhibited the activation of ERKs. In contrast, treatment of IPDE4 alone had little impacts. Consistent with this observation, combinational inhibition of PDE3 and PDE4 significantly inhibited cell growth elicited by PDGF (Figure 6c). Interestingly, IPDE3 alone, which had little effects on intracellular cAMP, PKA and activity of CRE and expression of Cx43, also caused significant inhibition of mesangial cell growth. It was contrastive to the lack of significant inhibition by IPDE4.

Previous reports showed that the cAMP pathway regulates behavior of mesangial cells including cell shape and apoptosis (Kreisberg *et al*., 1984; Glass *et al*., 1988; Muhl *et al*., 1996). We next examined roles of PDE3 and PDE4 in the regulation of mesangial cell survival. Reporter cells were serum-depleted and treated with IPDE3, IPDE4, IPDE3 plus IPDE4, IPDE2 plus IPDE5 or IBMX for 4–12 h. Microscopic analyses revealed that treatment of the cells with IPDE3 plus IPDE4 induced dramatic morphological alteration from the spindle to retracted shape (Figure 7a). This morphological change occurred within 1 h and peaked at 3–5 h, which was mimicked by the nonselective inhibitor of PDEs, IBMX. In contrast, none of IPDE3, IPDE4, or IPDE2 plus IPDE5 induced any morphologic changes. Similarly, inhibition of any other single PDE or other combinational inhibition of PDEs did not induce morphological alterations (data not shown). Hoechst staining showed that the morphological change of the cells was associated with nuclear condensation typical of apoptosis (Figure 7b). Quantitative analysis revealed that the number of apoptotic cells significantly increased from $2.00\pm0.82\%$ (untreated control) to $23.75\pm3.30\%$ by the concomitant inhibition of PDE3 and PDE4 (Figure 7c). Apoptosis was also induced to the same magnitude by the treatment with IBMX. Furthermore, Western blot analysis revealed that caspase-3 was activated by IPDE3 plus IPDE4 or IBMX, but not by IPDE3 or IPDE4 alone (Figure 7d). IPDE2 plus IPDE5 also had little impacts on the cleavage of procaspase-3.

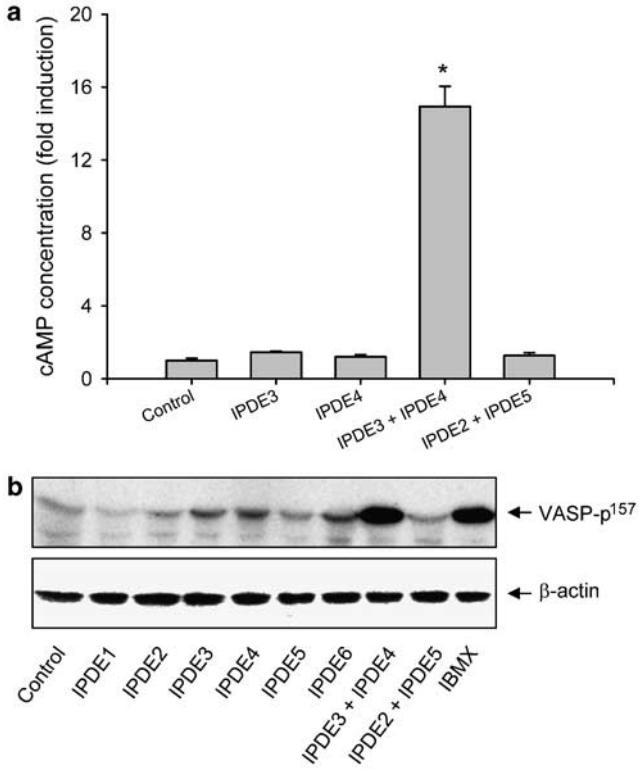

Figure 4 Induction of intracellular cAMP and activation of PKA by inhibition of PDE3 and PDE4. (a) Effects of PDE inhibitors on the level of cAMP. Mesangial cells were treated with IPDE3, IPDE4, IPDE3 plus IPDE4, or IPDE2 plus IPDE5 for 1 h, and cellular extracts were subjected to assays for intracellular cAMP. Data were shown as means ± s.e. and an asterisk indicates a statistically significant difference ($P<0.01$). (b) Phosphorylation of VASP, an indicator for PKA activation, by PDE inhibitors. Mesangial cells were treated with IPDEs individually or in indicated combinations for 1 h, and cellular proteins were subjected to Western blot analysis of phosphorylated VASP at serine 157. The level of β-actin was shown as a loading control.

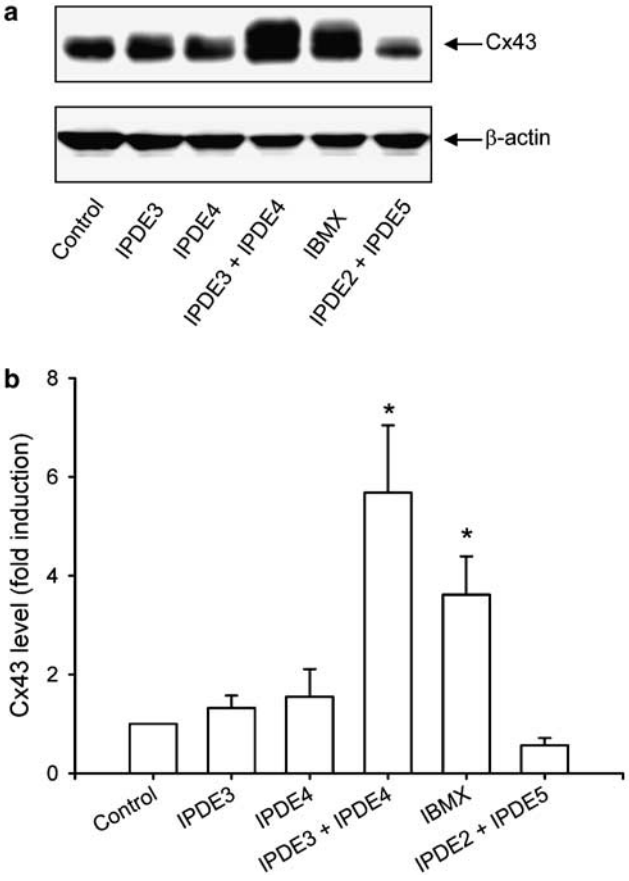

Figure 5 Induction of Cx43 by inhibition of IPDE3 and IPDE4. Mesangial cells were treated with IPDE3, IPDE4, IPDE3 plus IPDE4, IBMX or IPDE2 plus IPDE5 for 24 h and subjected to Western blot analysis of Cx43 (a). Densitometric analysis of the data is shown in (b). Asterisks indicate statistically significant differences ($P<0.05$).

Activation of the cAMP pathway by cGMP-mediated inhibition of PDE3

cGMP is known to be a potent inhibitor of PDE3 (Osinski *et al*., 2001). In mesangial cells, treatment with 8-Br-cGMP significantly induced activation of the cAMP pathway (Figure 1f). To confirm that the effect of cGMP was *via* inhibition of PDE3, we compared effects of three different cGMP-elevating agents including nitric oxide donor *S*-nitroso-*N*-acetylpenicillamine (SNAP), 8-Br-cGMP and 8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphate (8-pCPT-cGMP) on the activation of CRE. Like 8-Br-cGMP, 8-pCPT-cGMP is known to activate protein kinase G but does not interact with PDE3 (Osinski *et al*., 2001). As shown in Figure 8a, SNAP, 8-Br-cGMP and 8-pCPT-cGMP mimicked the promoting effect of IPDE3 on the cAMP-induced activation of CRE. However, the effect of 8-pCPT-cGMP that does not inhibit PDE3 was significantly lower than that of 8-Br-cGMP. The difference between 8-Br-cGMP and 8-pCPT-cGMP was observed at all concentrations tested ranging from 10 to 500 μM (Figure 8b). In the presence of IPDE4, activation of the cAMP pathway by IPDE3 was mimicked by 8-Br-cGMP (Figure 8c). Similarly, in the presence of 8-Br-cAMP, enhanced activation of CRE by IPDE3 was reproduced by the treatment with 8-Br-cGMP. Additional treatment with IPDE3 did not further enhance activation of CRE by 8-Br-cGMP plus 8-Br-cAMP (Figure 8c). These results suggested that cGMP induced activation of the cAMP pathway, at least in part, *via* inhibition of PDE3 in mesangial cells.

Discussion

cAMP is an important intracellular second messenger responsible for a variety of cellular responses to external stimuli. Most of the biological actions of cAMP are mediated by activation of PKA. In many cases, the cAMP pathway generates negative/inhibitory signals that counteract positive/stimulatory signals triggered by cytokines and growth factors (Dousa, 1998; 1999). Because of this reason, previous studies demonstrated beneficial effects of cAMP-elevating agents, for example, inhibitors of PDEs, on the treatment of various diseases (Dousa, 1998; 1999). However, therapeutic utility of nonselective PDE inhibitors may be limited due to their systemic, unfavorable effects. For the purpose of therapeutic intervention, use of selective PDE inhibitors targeting particular cell types will be essential. Profiling of functional PDEs

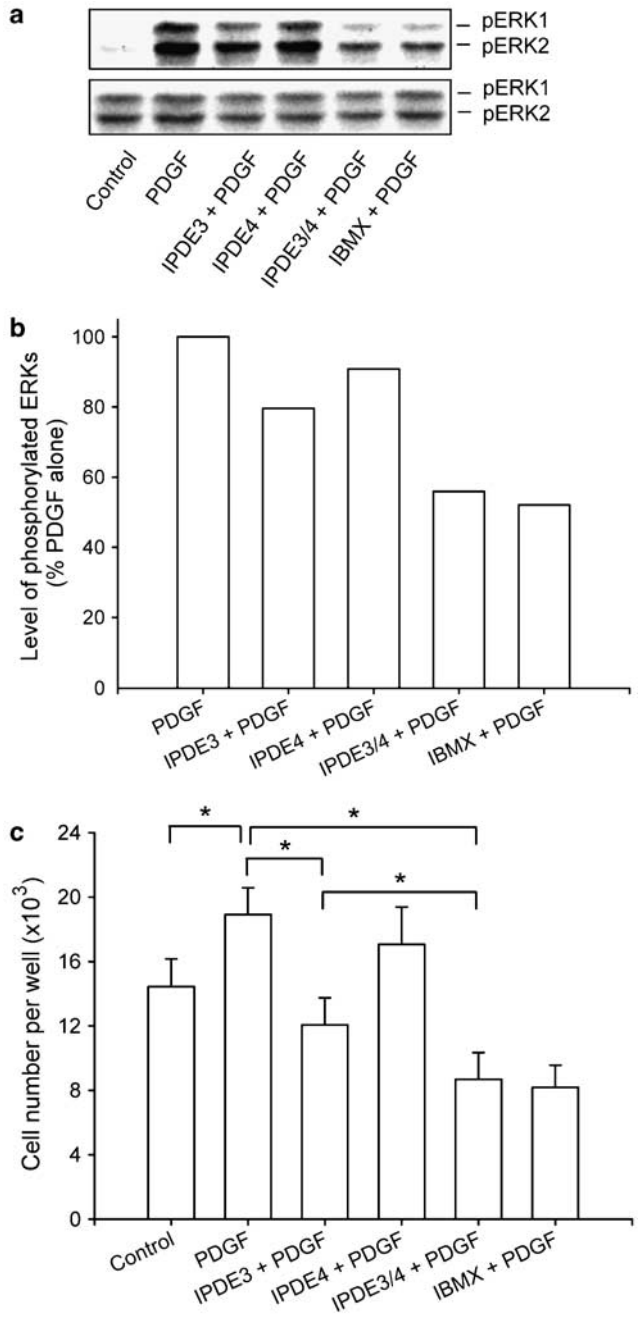

Figure 6 Effects of PDE inhibition on PDGF-induced activation of extracellular signal-regulated kinase (ERK) and cell proliferation. (a) Mesangial cells were pretreated with IPDE3, IPDE4, IPDE3 plus IPDE4, or IBMX for 1 h, and exposed to 20 ng ml^{-1} PDGF for 5 min. Cellular proteins were subjected to Western blot analysis of phosphorylated ERK1 (pERK1) and ERK2 (pERK2). Protein levels of ERKs were shown at the bottom as loading controls. Densitometric analysis of the data is shown in (b). (c) Mesangial cells seeded in 96-well culture plates were treated with PDGF in the presence of IPDE3, IPDE4, IPDE3 plus IPDE4, or IBMX in the presence of PDGF for 48 h, and the number of cells was evaluated. Data were shown as means ± s.e. and asterisks indicate statistically significant differences.

in individual tissues and cell types is, therefore, required for the development of effective therapies. In the present report, we attempted profiling of PDEs in the control of mesangial cell function. Using reporter mesangial cells that secrete SEAP under the control of CRE, we examined effects of selective inhibitors for individual PDEs on the activity of the cAMP pathway under basal and stimulated conditions. We found that treatment of mesangial cells with the nonselective PDE inhibitor IBMX markedly induced activation of CRE, suggesting that PDEs play crucial roles in the suppression of the cAMP pathway in unstimulated mesangial cells. However, treatment with individual PDE inhibitor did not induce activation of CRE. Subsequent experiments revealed that, among different combinations tested, concomitant inhibition of PDE3 and PDE4 exclusively and markedly increased intracellular cAMP and PKA activity, triggered activation of CRE and caused induction of CRE-regulated protein Cx43. Our results elucidated that cooperation of PDE3 and PDE4 plays a crucial role in the regulation of the cAMP pathway and downstream gene expression in mesangial cells.

In contrast to the response under the basal culture condition, inhibition of PDE2, PDE3, PDE4 or PDE5 significantly enhanced activation of CRE under the 8-bromo-cAMP- or 8-bromo-cGMP-stimulated conditions. These results indicated distinct roles of individual PDEs in mesangial cells under unstimulated and stimulated conditions. That is, under unstimulated situations, only PDE3 and PDE4 cooperatively regulate mesangial cell function, whereas under stimulated conditions, PDE2, PDE3, PDE4 and PDE5 can exert substantial regulatory effects individually and independently. In contrast to PDE2–PDE5, we did not obtain any evidence that PDE1 and PDE6 were functional in mesangial cells under either unstimulated or stimulated conditions.

Given PDE2 and PDE5 can be activated by the increase in cGMP (Dousa, 1998), the pronounced activation of CRE by IPDE2 or IPDE5 in the presence of cGMP indicates existence of functional PDE2 and PDE5 in mesangial cells. These enzymes, once being activated, may play important roles in the regulation of cAMP signaling.

One of major findings in the present study is the dramatic activation of the cAMP pathway by concomitant inhibition of PDE3 and PDE4. It was closely correlated with the induction of the major gap junction protein Cx43. In general, gap junction is critically involved in the regulation of cell growth, migration, survival and differentiation (Goodenough *et al*., 1996; Kumar & Gilula, 1996). In mesangial cells, Cx43 may be involved in the suppression of mitogenesis and induction of differentiation (Yao *et al*., 2000; 2006). For example, short-term exposure of mesangial cells to PDGF caused rapid and reversible inhibition of gap junctional communication *via* phosphatidylinositol 3-kinase (Yao *et al*., 2000), which is also involved in PDGF-induced mitogenesis of mesangial cells (Choudhury *et al*., 1994). Recently, we also demonstrated that long-term exposure of mesangial cells to PDGF (in the presence of cAMP-elevating agents) significantly increased the level of Cx43 and that it was accompanied by reduction of α-smooth muscle actin, a de-differentiation marker of mesangial cells (Yao *et al*., 2006). These previous data, together with our current findings, suggested a possibility that PDE3 and PDE4 may cooperatively regulate mesangial cell mitogenesis and differentiation under unstimulated situations. Indeed, simultaneous inhibition of PDE3 and PDE4 attenuated PDGF-induced activation of ERKs and abrogated PDGF-induced mesangial cell proliferation, as shown in this report. It is consistent with previous reports showing the involvement of PDE3 and PDE4 in the mitogenesis of mesangial

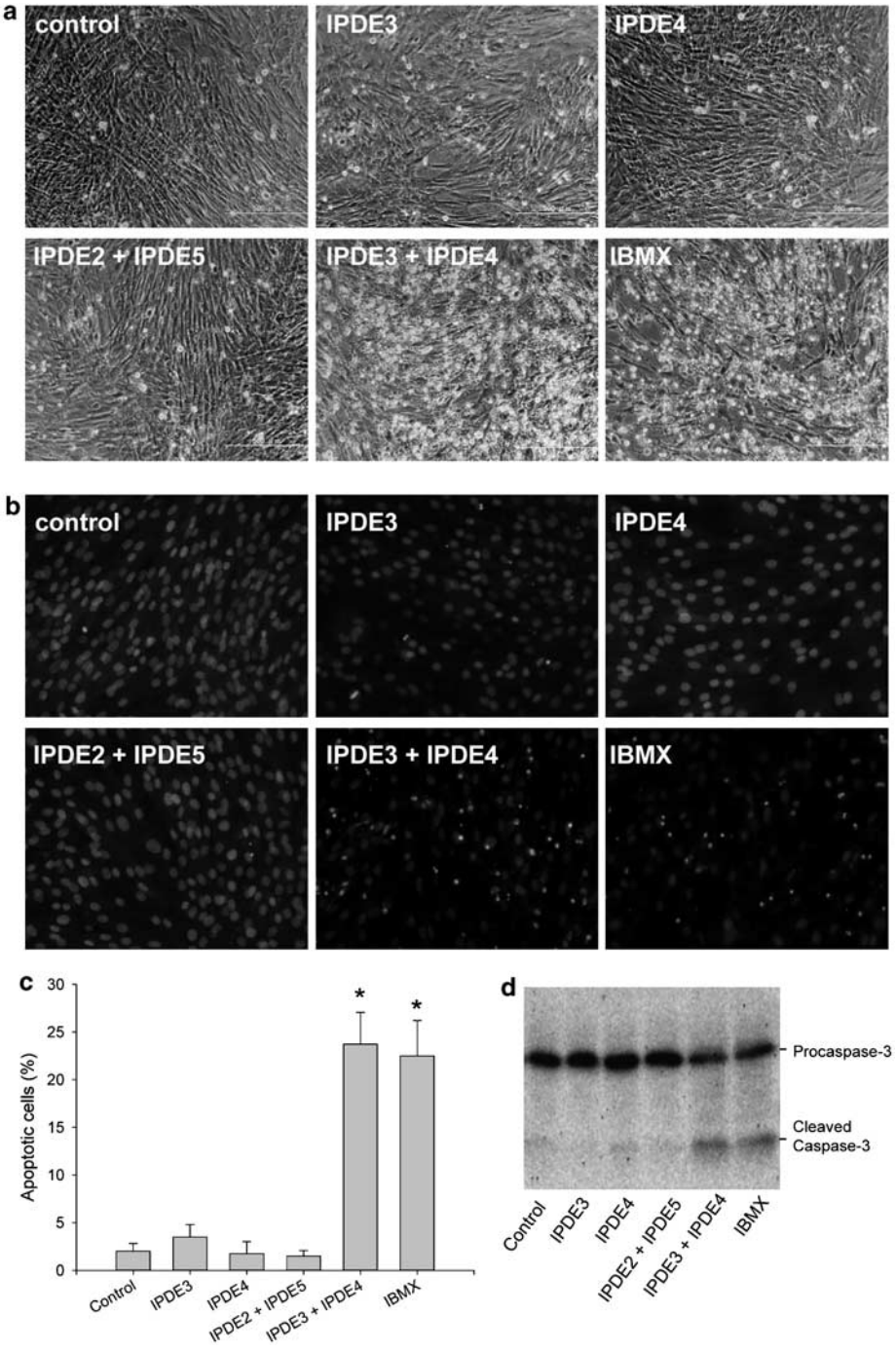

Figure 7 Regulation of cell survival by PDE3 and PDE4. Mesangial cells in serum-free medium were treated with IPDE3, IPDE4, IPDE3 plus IPDE4, IPDE2 plus IPDE5, or IBMX for 4–12 h and subjected to phase-contrast microscopy (a), Hoechst33258 staining (b, c) and Western blot analysis of caspase-3 (d). Percentages of apoptotic cells were assessed quantitatively by fluorescence microscopy (c). Data were expressed as means $\pm$ s.e. and asterisks indicate statistically significant differences ($P<0.01$). In (d), the top band represents procaspase-3 (M_r 35,000), and the bottom band indicates its cleaved, mature form (M_r 17,000).

cells (Matousovic *et al*., 1995; Dousa, 1998; Cheng *et al*., 2004). Interestingly, IPDE3 alone, which had little influences on cAMP, PKA, CRE and Cx43, also caused significant inhibition of mesangial cell proliferation, which was not observed by IPDE4. The differential regulation of mesangial cell proliferation by PDE3 and PDE4 inhibitors has also been reported by Cheng *et al*. (2004). These inhibitors have the different potential for suppression of the Ras–Raf–MAP kinase pathway because of different compartmentalization of cAMP pools. It is, however, still unclear why suppression of mesangial cell growth by IPDE3 was not accompanied by obvious increase in Cx43, the level of which is inversely correlated with cell proliferation (Goodenough *et al*., 1996; Kumar & Gilula, 1996). Further investigation will be required to clarify mechanisms involved in this phenomenon.

The cAMP pathway is known to regulate survival of various cells. cAMP has been regarded as a bifunctional regulator of apoptosis; for example, cAMP induces apoptosis in lymphoid cells, whereas it protects other cell types (e.g., neutrophils and eosinophils) from drug- or cytokine-triggered apoptosis (Roger *et al*., 1995; Chang *et al*., 2000; Usher *et al*., 2002). Similarly, both pro- and anti-apoptotic potential of cAMP has been reported in mesangial cells (Muhl *et al*., 1996; Manderscheid *et al*., 2002). We found that, under serum deprivation, inhibition of PDEs by a nonselective inhibitor IBMX led to morphological changes and apoptosis, and it was mimicked exclusively by concomitant inhibition of PDE3 and PDE4. This result suggested that, in addition to their effects on cell proliferation and differentiation, PDE3 and PDE4 may also be involved in the antiapoptotic machinery that supports survival of mesangial cells.

In the present study, we identified PDE3 and PDE4 as the major PDEs in the regulation of mesangial cell function. However, as shown in Figure 2b, IPDE3, but not IPDE4, strongly activated the cAMP pathway in the presence of 8-Br-cAMP. Previous reports showed that, although PDE4 was more potent in hydrolysis of cAMP than PDE3, inhibition of PDE3 induced higher activation of PKA than that by inhibition of PDE4 (Matousovic *et al*., 1995; Chini *et al*., 1997a; Dousa, 1998; 1999). This may be due to the fact that different cAMP pools are present in mesangial cells, in which the PDE3-regulated cAMP pool is more closely associated with activation of PKA (Matousovic *et al*., 1995; Chini *et al*., 1997a; Dousa, 1998; 1999). The distinct cooperation of IPDE3 and IPDE4 with 8-Br-cAMP, demonstrated in this report, might be explained by this concept. In addition, cAMP induced by IPDE3 or IPDE4 could be highly compartmentalized and insufficient to increase total cellular cAMP (Aizawa *et al*., 2003). This may explain the failure to detect a significant change in cAMP after single inhibition of PDE3 or PDE4.

cGMP is known to activate the cAMP pathway *via* inhibiting PDE3 and/or direct activation of PKA at high doses (Sausbier *et al*., 2000; Osinski *et al*., 2001). Using the CRE-SEAP reporting system, we demonstrated that (1) cGMP-elevating agents induced activation of the cAMP pathway, (2) compared with 8-Br-cGMP, 8-pCPT-cGMP, a cGMP analogue that does not interact with PDE3, was less effective, (3) the effect of IPDE3 was reproduced by 8-Br-cGMP, and (4) IPDE3 did not enhance activation of the cAMP pathway by 8-Br-cGMP. These results revealed that cGMP induced activation of the cAMP pathway, at least in part, *via* inhibition of PDE3 in mesangial cells. However, mechanisms other than PDE3 inhibition should also be

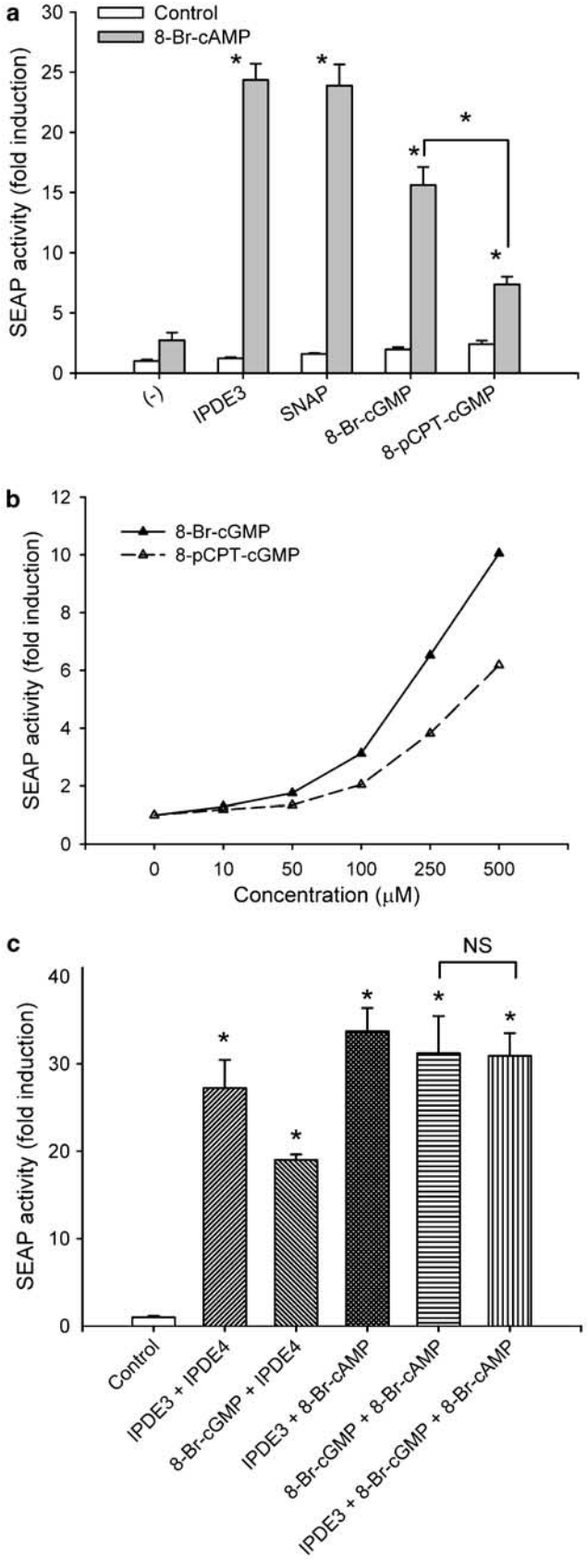

Figure 8 Activation of the cAMP pathway by cGMP-mediated inhibition of PDE3. (a) Effects of cGMP-elevating agents on the activation of CRE. Reporter cells were treated with IPDE3 or cGMP-elevating agents including 150 μM SNAP, 500 μM 8-Br-cGMP and 500 μM 8-pCPT-cGMP in the absence (Control) or presence of 500 μM 8-Br-cAMP for 24 h. Activity of SEAP was evaluated by chemiluminescence assay. (b) Dose-dependent effects of 8-Br-cGMP and 8-pCPT-cGMP on the activation of CRE. Reporter cells were treated with 8-Br-cGMP or 8-pCPT-cGMP at indicated concentrations in the presence of 500 μM 8-Br-cAMP for 24 h, and activity of SEAP was evaluated. (c) Combinational effects of IPDEs, cAMP and cGMP on the activation of CRE. Reporter cells were treated with IPDE3, IPDE4, 500 μM 8-Br-cAMP and 1000 μM 8-Br-cGMP using indicated combinations for 24 h and subjected to SEAP assay. Data were expressed as means $\pm$ s.e. and asterisks indicate statistically significant differences. NS, not significant.

involved in the cGMP-elicited CRE activation, because (1) 8-pCPT-cGMP, which does not interact with PDE3, also triggered significant activation of CRE, and (2) inhibition of the major cGMP-degradating enzymes, PDE5 and PDE2, also significantly potentiated the effect of cGMP on CRE. Because IPDE3 did not fully activate CRE even at high concentrations, inhibition of PDE3 by cGMP alone may not be sufficient for induction of CRE activation.

Previous reports showed that the cGMP signaling pathway is involved in the regulation of mesangial cell function (Garg & Hassid, 1989; Craven *et al*., 1997; Yao *et al*., 1998). cGMP-elevating agents, such as nitric oxide, exert multiple biological effects on mesangial cells (Garg & Hassid, 1989; Craven *et al*., 1997; Yao *et al*., 1998). Our results suggested that activation of the cAMP pathway *via* inhibition of PDE3 could be involved in the effects of cGMP-elevating agents on mesangial cells. The CRE-SEAP-based reporter system may provide a convenient and useful tool to further analyze and elucidate interactions between the cAMP and cGMP signaling pathways.

Activity of PDEs is usually evaluated by analyzing ability of cell lysates to hydrolyze specific nucleotide (cAMP or cGMP) in the presence or absence of specific PDE inhibitors. The levels of PDE isozymes can also be evaluated by Northern and Western blot analyses. In contrast to these conventional approaches, however, the SEAP-based reporter system has several advantages. First, this system is able to assess and track activity of PDEs in living cells. Second, the system is based on a chemiluminescence assay that allows for simple, rapid, sensitive and quantitative evaluation (Kasai *et al*, 2004; 2005). Third, small-scale assays using 96-well or 384-well plates are feasible because only 5 μl of culture medium is required for analysis. This property allows for economical screening of bioactive substances that affect activity of PDEs using small numbers of reporter cells. Finally, because preparation of cell lysates is not required, continuous assessment using identical cell cultures is possible by serial sampling of culture medium (Meng *et al*., 2005a). Taken together, the system demonstrated in this report would provide a powerful tool for profiling of functional PDEs in individual tissues and cell types, for large-scale screening of therapeutic drugs that affect the level of cAMP, and for elucidation of interactions among various molecules involved in the regulation of the cAMP signaling pathway.

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (16390243 to M.K. and 17659255 to J.Y.).

References

AIZAWA, T., WEI, H., MIANO, J.M., ABE, J., BERK, B.C. & YAN, C. (2003). Role of phosphodiesterase 3 in NO/cGMP-mediated anti-inflammatory effects in vascular smooth muscle cells. *Circ. Res.,* **93,** 406–413.

BAILEY, J., PHILLIPS, R.J., POLLARD, A.J., GILMORE, K., ROBSON, S.C. & EUROPE-FINNER, G.N. (2002). Characterization and functional analysis of cAMP response element modulator protein and activating transcription factor 2 (ATF2) isoforms in the human myometrium during pregnancy and labor: identification of a novel ATF2 species with potent transactivation properties. *J. Clin. Endocrinol. Metab.,* **87,** 1717–1728.

BEAVO, J.A. (1995). Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiol. Rev.,* **75,** 725–748.

BEGANY, D.P., CARCILLO, J.A., HERZER, W.A., MI, Z. & JACKSON, E.K. (1996). Inhibition of type IV phosphodiesterase by Ro20-1724 attenuates endotoxin-induced acute renal failure. *J. Pharmacol. Exp. Ther.,* **278,** 37–41.

BERGER, J., HAUBER, J., HAUBER, R., GEIGER, R. & CULLEN, B.R. (1988). Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukaryotic cells. *Gene,* **66,** 1–10.

CHANG, H.S., JEON, K.W., KIM, Y.H., CHUNG, I.Y. & PARK, C.S. (2000). Role of cAMP-dependent pathway in eosinophil apoptosis and survival. *Cell. Immunol.,* **203,** 29–38.

CHANSON, M., WHITE, M.M. & GARBER, S.S. (1996). cAMP promotes gap junctional coupling in T84 cells. *Am. J. Physiol.,* **271,** C533–C539.

CHENG, J., THOMPSON, M.A., WALKER, H.J., GRAY, C.E., DIAZ ENCARNACION, M.M., WARNER, G.M. & GRANDE, J.P. (2004). Differential regulation of mesangial cell mitogenesis by cAMP phosphodiesterase isozymes 3 and 4. *Am. J. Physiol. Renal. Physiol.,* **287,** F940–F953.

CHINI, E.N., CHINI, C.C., BOLLIGER, C., JOUGASAKI, M., GRANDE, J.P., BURNETT Jr, J.C. & DOUSA, T.P. (1997b). Cytoprotective effects of adrenomedullin in glomerular cell injury: central role of cAMP signaling pathway. *Kidney Int.,* **52,** 917–925.

CHINI, C.C., CHINI, E.N., WILLIAMS, J.M., MATOUSOVIC, K. & DOUSA, T.P. (1994). Formation of reactive oxygen metabolites in glomeruli is suppressed by inhibition of cAMP phosphodiesterase isozyme type IV. *Kidney Int.,* **46,** 28–36.

CHINI, C.C., GRANDE, J.P., CHINI, E.N. & DOUSA, T.P. (1997a). Compartmentalization of cAMP signaling in mesangial cells by phosphodiesterase isozymes PDE3 and PDE4. Regulation of superoxidation and mitogenesis. *J. Biol. Chem.,* **272,** 9854–9859.

CHOUDHURY, G.G., BISWAS, P., GRANDALIANO, G., FOUQUERAY, B., HARVEY, S.A. & ABBOUD, H.E. (1994). PDGF-mediated activation of phosphatidylinositol 3 kinase in human mesangial cells. *Kidney Int.,* **46,** 37–47.

CRAVEN, P.A., STUDER, R.K., FELDER, J., PHILLIPS, S. & DERUBERTIS, F.R. (1997). Nitric oxide inhibition of transforming growth factor-beta and collagen synthesis in mesangial cells. *Diabetes,* **46,** 671–681.

CULLEN, B.R. & MALIM, M.H. (1992). Secreted placental alkaline phosphatase as a eukaryotic reporter gene. *Methods Enzymol.,* **216,** 362–368.

DOUSA, T.P. (1998). Signaling role of PDE isozymes in pathobiology of glomerular mesangial cells. Studies *in vitro* and *in vivo*. *Cell Biochem. Biophys.,* **29,** 19–34.

DOUSA, T.P. (1999). Cyclic-3′,5′-nucleotide phosphodiesterase isozymes in cell biology and pathophysiology of the kidney. *Kidney Int.,* **55,** 29–62.

DUFAU, M.L., TSURUHARA, T., HORNER, K.A., PODESTA, E. & CATT, K.J. (1977). Intermediate role of adenosine 3′:5′-cyclic monophosphate and protein kinase during gonadotropin-induced steroidogenesis in testicular interstitial cells. *Proc. Natl. Acad. Sci. U.S.A.,* **74,** 3419–3423.

GARG, U.C. & HASSID, A. (1989). Inhibition of rat mesangial cell mitogenesis by nitric oxide-generating vasodilators. *Am. J. Physiol.,* **257,** F60–F66.

GLASS II, W.F., RADNIK, R.A., GARONI, J.A. & KREISBERG, J.I. (1988). Urokinase-dependent adhesion loss and shape change after cyclic adenosine monophosphate elevation in cultured rat mesangial cells. *J. Clin. Invest.,* **82,** 1992–2000.

GOODENOUGH, D.A., GOLIGER, J.A. & PAUL, D.L. (1996). Connexins, connexons, and intercellular communication. *Annu. Rev. Biochem.,* **65,** 475–502.

HIRAMATSU, N., KASAI, A., MENG, Y., HAYAKAWA, K., YAO, J. & KITAMURA, M. (2005). Alkaline phosphatase vs luciferase as secreted reporter molecules *in vivo*. *Anal. Biochem.,* **339,** 249–256.

HIRAMATSU, N., KASAI, A., YAO, J., MENG, Y., TAKEDA, M., MAEDA, S. & KITAMURA, M. (2004). AP-1-independent sensitization to oxidative stress-induced apoptosis by proteasome inhibitors. *Biochem. Biophys. Res. Commun.,* **316,** 545–552.

HOUSLAY, M.D. & MILLIGAN, G. (1997). Tailoring cAMP-signalling responses through isoform multiplicity. *Trends Biochem. Sci.,* **22,** 217–224.

ISHIKAWA, Y. & KITAMURA, M. (2000). Anti-apoptotic effect of quercetin: intervention in the JNK- and ERK-mediated apoptotic pathways. *Kidney Int.,* **58,** 1078–1087.

KASAI, A., HIRAMATSU, N., MENG, Y., YAO, J., MAEDA, S. & KITAMURA, M. (2005). Fast-track DRESSA: a bioassay for fast, sensitive, and selective detection of halogenated and polycyclic aromatic hydrocarbons. *Anal. Biochem.,* **337,** 84–88.

KASAI, A., HIRAMATSU, N., MENG, Y., YAO, J., TAKEDA, M., MAEDA, S. & KITAMURA, M. (2004). DRESSA: biosensing of dioxin and dioxin-like chemicals using secreted alkaline phosphatase. *Anal. Biochem.,* **335,** 73–80.

KITAMURA, M., SUTO, T., YOKOO, T., SHIMIZU, F. & FINE, L.G. (1996). Transforming growth factor-β1 is the predominant paracrine inhibitor of macrophage cytokine synthesis produced by glomerular mesangial cells. *J. Immunol.,* **156,** 2964–2971.

KITAMURA, M., TAYLOR, S., UNWIN, R., BURTON, S., SHIMIZU, F. & FINE, L.G. (1994). Gene transfer into the rat renal glomerulus *via* a mesangial cell vector: site-specific delivery, *in situ* amplification, and sustained expression of an exogenous gene *in vivo*. *J. Clin. Invest.,* **94,** 497–505.

KREISBERG, J.I., VENKATACHALAM, M.A. & PATEL, P.Y. (1984). Cyclic AMP-associated shape change in mesangial cells and its reversal by prostaglandin E2. *Kidney Int.,* **25,** 874–879.

KUMAR, N.M. & GILULA, N.B. (1996). The gap junction communication channel. *Cell,* **84,** 381–388.

MANDERSCHEID, M., PEREDA-FERNANDEZ, C. & PFEILSCHIFTER, J. (2002). Cyclic AMP increases rat inhibitor of apoptosis protein (RIAP1) mRNA in renal mesangial cells. *Kidney Int.,* **61,** 797–803.

MATOUSOVIC, K., GRANDE, J.P., CHINI, C.C., CHINI, E.N. & DOUSA, T.P. (1995). Inhibitors of cyclic nucleotide phosphodiesterase isozymes type-III and type-IV suppress mitogenesis of rat mesangial cells. *J. Clin. Invest.,* **96,** 401–410.

MENG, Y., KASAI, A., HIRAMATSU, N., HAYAKAWA, K., TAKEDA, M., SHIMIZU, F., KAWACHI, H., YAO, J. & KITAMURA, M. (2005a). Real-time monitoring of mesangial cell-macrophage cross-talk using SEAP *in vitro* and *ex vivo*. *Kidney Int.,* **68,** 886–893.

MENG, Y., KASAI, A., HIRAMATSU, N., HAYAKAWA, K., YAMAUCHI, K., TAKEDA, M., KAWACHI, H., SHIMIZU, F., YAO, J. & KITAMURA, M. (2005b). Continuous, noninvasive monitoring of local microscopic inflammation using a genetically engineered cell-based biosensor. *Lab Invest,* **85,** 1429–1439.

MUHL, H., NITSCH, D., SANDAU, K., BRUNE, B., VARGA, Z. & PFEILSCHIFTER, J. (1996). Apoptosis is triggered by the cyclic AMP signalling pathway in renal mesangial cells. *FEBS Lett.,* **382,** 271–275.

OSINSKI, M.T., RAUCH, B.H. & SCHROR, K. (2001). Antimitogenic actions of organic nitrates are potentiated by sildenafil and mediated *via* activation of protein kinase A. *Mol. Pharmacol.,* **59,** 1044–1050.

ROGER, P.P., REUSE, S., MAENHAUT, C. & DUMONT, J.E. (1995). Multiple facets of the modulation of growth by cAMP. *Vitam. Horm.,* **51,** 59–191.

SAUSBIER, M., SCHUBERT, R., VOIGT, V., HIRNEISS, C., PFEIFER, A., KORTH, M., KLEPPISCH, T., RUTH, P. & HOFMANN, F. (2000). Mechanisms of NO/cGMP-dependent vasorelaxation. *Circ. Res.,* **87,** 825–830.

SHULZ, P.J., DICORLETO, P.E., SILVER, B.J. & ABBOUD, H.E. (1988). Mesangial cells express PDGF mRNAs and proliferate in response to PDGF. *Am. J. Physiol.,* **255,** F674–F684.

TAM, F.W., SMITH, J., AGARWAL, S., KARKAR, A.M., MOREL, D., THOMPSON, E.M. & PUSEY, C.D. (2000). Type IV phosphodiesterase inhibitor is effective in prevention and treatment of experimental crescentic glomerulonephritis. *Nephron,* **84,** 58–66.

TENBROEK, E.M., LAMPE, P.D., SOLAN, J.L., REYNHOUT, J.K. & JOHNSON, R.G. (2001). Ser364 of connexin43 and the upregulation of gap junction assembly by cAMP. *J. Cell Biol.,* **155,** 1307–1318.

TSUBOI, Y., SHANKLAND, S.J., GRANDE, J.P., WALKER, H.J., JOHNSON, R.J. & DOUSA, T.P. (1996). Suppression of mesangial proliferative glomerulonephritis development in rats by inhibitors of cAMP phosphodiesterase isozymes types III and IV. *J. Clin. Invest.,* **98,** 262–270.

USHER, L.R., LAWSON, R.A., GEARY, I., TAYLOR, C.J., BINGLE, C.D., TAYLOR, G.W. & WHYTE, M.K. (2002). Induction of neutrophil apoptosis by the *Pseudomonas aeruginosa* exotoxin pyocyanin: a potential mechanism of persistent infection. *J. Immunol.,* **168,** 1861–1868.

YAO, J., HIRAMATSU, N., ZHU, Y., MORIOKA, T., TAKEDA, M., OITE, T. & KITAMURA, M. (2005). Nitric oxide-mediated regulation of connexin43 expression and gap junction intercellular communication in mesangial cells. *J. Am. Soc. Nephrol.,* **16,** 58–67.

YAO, J., KITAMURA, M., ZHU, Y., MENG, Y., KASAI, A., HIRAMATSU, N., MORIOKA, T., TAKEDA, M. & OITE, T. (2006). Synergistic effects of PDGF-BB and cAMP-elevating agents on expression of connexin43 in mesangial cells. *Am. J. Physiol. Renal. Physiol.,* **290,** F1083–F1093.

YAO, J., MORIOKA, T. & OITE, T. (2000). PDGF regulates gap junction communication and connexin43 phosphorylation by PI 3-kinase in mesangial cells. *Kidney Int.,* **57,** 1915–1926.

YAO, J., SCHOECKLMANN, H.O., PROLS, F., GAUER, S. & STERZEL, R.B. (1998). Exogenous nitric oxide inhibits mesangial cell adhesion to extracellular matrix components. *Kidney Int.,* **53,** 598–608.

(Received February 23, 2006
Revised March 28, 2006
Accepted April 12, 2006
Published online 5 June 2006)