Angiotensinogen Gene Expression Is Stimulated by the cAMP-Responsive Element Binding Protein in Opossum Kidney Cells

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Abstract. It has been reported previously that the addition of isoproterenol or forskolin stimulates the expression of the angiotensinogen (ANG) gene in opossum kidney (OK) 27 cells, an OK cell line with a fusion gene containing the 5'-flanking regulatory sequence of the rat ANG gene fused with a human growth hormone (hGH) gene as a reporter, pOgh (ANG N-1498/+ 18), permanently integrated into their genomes. To investigate whether the effect of isoproterenol or forskolin on the expression of the ANG gene is mediated via the nuclear 43-kD cAMP-responsive element binding protein (CREB), OK 27 cells were transiently transfected with an expression plasmid containing the cDNA for the 43-kD CREB (pRSV/CREB). The level of expression of the pOgh (ANG N-1498/+18) in OK 27 cells was estimated by the amount of immunoreactive hGH secreted into the culture medium. Transfection of pRSV/CREB alone stimulated the expression of pOgh (ANG N-1498/+18). The addition of isoproterenol or forskolin further enhanced the stimulatory effect of pRSV/CREB on the expression of pOgh (ANG N-1498/+18). The enhancing effect of isoproterenol was inhibited by the presence of propranolol (an inhibitor of β-adrenoceptors) and (R)-p-adenosine 3'5'-cyclic monophospho-orthioate (Rp)-cAMP (an inhibitor of cAMP-dependent protein kinase A I and II). Transfection of pRSV/CREB had no effect on the expression of thymidine kinase growth hormone in OK 13 cells, an OK cell line with a fusion gene containing the promoter/enhancer DNA sequence of the viral thymidine-kinase gene fused with an hGH gene as a reporter, thymidine kinase growth hormone, permanently integrated into their genomes. These studies demonstrate that isoproterenol stimulates the expression of ANG gene via the cAMP-dependent protein kinase A and probably via the interaction of the 43-kD CREB with the 5'-flanking region of the ANG gene. Our data indicate that the nuclear 43-kD CREB may have a modulatory role on the expression of the ANG gene in OK cells. (J Am Soc Nephrol 8: 1072–1079, 1997)

Angiotensinogen (ANG) mRNA has been localized in rat renal proximal tubules by the techniques of in situ hybridization (1) and PCR (2). Furthermore, studies by Wolf and Neilson (3) and Tang et al. (4) showed that ANG mRNA is expressed in mouse and rat immortalized proximal tubular cell lines, respectively. We (5), as well as Ingelfinger et al. (6), have also demonstrated that the ANG mRNA is expressed in opossum kidney (OK) proximal tubular cells. Thus, these studies demonstrate that the intrarenal angiotensin (Ang) I and II are probably derived from the ANG synthesized in renal proximal tubules.

We have reported previously that thyroid hormone (L-T3), dexamethasone, 8-bromo-cAMP (8-Br-cAMP), and forskolin stimulate the expression of the ANG gene in OK cells in vitro in a dose-dependent manner (5,7,8). We have also demonstrated that isoproterenol stimulates the expression of the ANG gene in OK cells (9). The effect of isoproterenol is mediated via the β1-adrenoceptor and is blocked by the presence of propranolol (β-adrenoceptor blocker), atenolol (β1-adrenoceptor blocker), and (R)-p-adenosine 3'5'-cyclic monophospho-orthioate (Rp)-cAMP (an inhibitor of cAMP-dependent protein kinase A I and II) (9), but not by ICI 118,551 (β2-adrenoceptor blocker). The specificity of the β1-adrenoceptor is supported further by our more recent studies that isoproterenol stimulates the expression of the ANG gene when cotransfected with an expression vector containing the cDNA for the β1-adrenoceptor, but not the cDNA for the β2-adrenoceptor (10). Our studies confirm the report of Nakamura and Johns (11) that the administration of atenolol blocks the effect of renal nerve stimulation after the increase of the ANG mRNA level in the rat kidney in vivo. Furthermore, our studies (9,10) and those of Nakamura and Johns (11) together suggest the presence of a functional relationship between the renal sympathetic nervous system and the activation of local renal renin-angiotensin system. Thus, the local formation of renal Ang II may play an important role in the modulation of the physiology of the renal proximal tubular cells (i.e., sodium and fluid reabsorption) (12–17).

The exact molecular mechanism(s) for the effect of isoproterenol on the expression of the ANG gene in OK cells is unknown. One possibility may be that the addition of isopro-
isoproterenol stimulates the synthesis of intracellular cAMP, which we have demonstrated previously (9). The intracellular cAMP then binds to the regulatory subunit of cAMP-dependent protein kinase A (PKA) and releases the catalytic subunit of PKA. The free catalytic subunit of PKA then translocates into the nucleus and subsequently phosphorylates the nuclear cAMP-responsive element binding protein (CREB) (18) or CREB-like proteins. The phosphorylated CREB or CREB-like protein(s) then interacts with the putative cAMP-responsive element (CRE) (i.e., ANG N-795 to N-788, TGACGTAC) in the 5′-flanking region of the rat ANG gene (19) and enhances the expression of the ANG gene.

Thus, in the present studies, we investigated the possibility of whether the 43 kD-CREB will directly enhance expression of the ANG gene in OK cells. Our studies demonstrated that the transient gene transfection of the plasmid containing the cDNA for the 43-kD CREB (pRSV/CРЕB) stimulates the expression of the fusion gene pOGH (ANG N-1498/+18) in OK 27 cells (9), an OK cell line into which has been stably integrated the fusion gene pOGH (ANG N-1498/+18) containing the 5′-flanking regulatory sequence of the rat ANG gene fused with a human growth hormone (hGH) gene as a reporter. The addition of isoproterenol or forskolin further enhanced the stimulatory effect of pRSV/CРЕB on the expression of pOGH (ANG N-1498/+18). Finally, the addition of propranolol and Rp-cAMP blocked the enhancing effect of isoproterenol on the expression of the pOGH (ANG N-1498/+18) in OK 27 cells when transfected with pRSV/CРЕB.

Materials and Methods

Materials

The fusion gene pOGH (ANG N-1498/+18) containing the 5′-flanking sequence (1498 bp) upstream of the transcriptional site plus 18 bp of Exon I of the rat ANG gene fused with an hGH gene, has been described previously (19). The plasmid pRSV-Neo containing the coding sequence for Neomycin (Neo) with the Rous sarcoma virus (RSV) enhancer/promoter sequence in the 5′-end of the Neo gene was a gift from Dr. Teresa Wang (Department of Pathology, Stanford University, Stanford, CA). The plasmid pRK/GH containing the thymidine kinase (TK) enhancer/promoter sequence fused to the 5′ end of the hGH gene was purchased from the Nichols Institute of Diagnostics (La Jolla, CA). The plasmid pGEM-3 as well as the pTKCAT and pRSVCAT vectors containing the coding sequence for chloramphenicol acetyltransferase (CAT) with TK or RSV enhancer/promoter sequence in the 5′ end of the CAT gene, respectively, were a gift from Dr. Joel F. Habener (Laboratory of Molecular Endocrinology, Massachusetts General Hospital, Boston, MA).

The expression plasmid containing the cDNA for the 43-kD CREB, pRSV/CРЕB, was constructed as follows. Briefly, total cellular RNA was prepared from adult, male rat liver (Wistar, Kyoto, Japan) by guanidium isothiocyanate/cesium chloride gradient as described previously (5). Twenty micrograms of total RNA was used to synthesize the double-strand cDNA for CREB by the method of reverse transcription and amplification by PCR (Thermal Cycler) according to the manual supplied by the manufacturer (Perkin Elmer Cetus, Inc., Foster City, CA). The nucleotide sequences for two oligonucleotide primers corresponding to the first 18 nucleotides (5′-ATG ACC ATG GAC TCT GGA-3′) and the last 18 nucleotides (5′-TTA ATC TGA CTT GTG GCA-3′) in the opening reading frame of the rat CREB (18), respectively, were used in PCR. The oligonucleotides were synthesized by Biosynthesis, Inc. (Lewisville, TX). One-kilobase CREB cDNA fragment was amplified and then subcloned in the plasmid pBlueScript (SK-) (Stratagene, Inc., La Jolla, CA). The sequence for the CREB cDNA was confirmed by dye-exchange sequencing with T3 and T7 primers (Stratagene, Inc.). Finally, the CREB cDNA containing the entire coding sequence was isolated and inserted into a mammalian cell expression vector pRC/RSV (purchased from Invitrogen, Inc., San Diego, CA). The plasmid pRC/RSV contains the RSV/long terminal repeat promoter and enhancer plus a bovine growth hormone polyadenylation signal for polyadenylation of transcribed mRNA.

The RIA kit for hGH (RIA-hGH) was a gift from National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health. The RIA procedure has been described previously (19). National Institute of Arthritis, Metabolism, and Digestive Diseases-hGH-I-1 (AFP-4793 B) was used for both iodination and as a hormone standard. The limit of sensitivity of the assay was 0.1 ng/ml. The inter- and intra-assay coefficients of variation were 10% (n = 10) and 12% (n = 10), respectively.

R(-)-isoproterenol (+)-bitartrate salt, forskolin, S(-)-propranolol hydrochloride, and Rp-cAMP (an inhibitor of the cAMP-dependent protein kinase A I and II) (20) were all purchased from Research Biochemicals, Inc. (Natick, MA).

Na-125I was purchased from DuPont, New England Nuclear (Boston, MA). Calcium chloride was purchased from Mallinckrodt, Inc. (Montreal, Quebec, Canada), and Geneticin (G 418) was purchased from Bethesda Research Laboratories (Gibco-BRL, Burlington, Ontario, Canada). Other molecular biology grade reagents were obtained either from Sigma (St. Louis, MO), Gibco-BRL, Boehringer-Mannheim (Dorval, Quebec, Canada), Pharmacia, Inc. (Baie d’Urfe, Quebec, Canada) or Promega-Fisher, Inc. (Montreal, Quebec, Canada).

Cell Culture

The OK proximal tubular cell line was obtained from the American Tissue Culture Collection (Rockville, MD). This cell line is derived from the kidney of a female American opossum, retains several properties of proximal tubular epithelial cells in culture (21,22), and expresses a low level of ANG mRNA (5–6). The culture conditions of OK cells have been described previously (5,7–10).

OK Cell Stable Transformants

Clones OK 27 and OK 13 are stable transformants with pOGH (ANG N-1498/+18) and pTKGH integrated into OK cellular genomes, respectively. The characteristics of these clones have been reported previously (9). Briefly, these clones were grown in the medium containing 500 μg/ml G 418 for more than 3 mo and expressed a high amount of immunoreactive (IR)-hGH into the medium. The expression of pOGH (ANG N-1498/+18) and pTKGH in OK 27 and OK 13 cells, respectively, was time-dependent. The levels of IR-hGH in cellular extracts were consistently less than 5% of that found in the media, indicating that IR-hGH is not stored in the cell (9).

DNA Transfection

The method of transient gene transfection into OK 27 and OK 13 cells is similar to that described previously in OK cells (7–10). Briefly, cells were incubated in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) immediately after transfection. Twenty-four hours after the DNA transfection, the media were replaced with fresh media without FBS. The media and cells
were harvested 24 h later and assayed for IR-hGH and CAT activity, respectively.

To study the effect of isoproterenol or forskolin on the expression of the fusion gene (pOOGH (ANG N-1498/+18)) in OK 27 cells transfected with pRSV/CREB, cells were incubated in DMEM containing 1% depleted FBS, and various concentrations of hormones were added on day 1 after DNA transfection. After a 24-h incubation period, the media and cells were harvested for hGH assays and CAT enzymatic assays, respectively. The control plasmid pRC/RSV was used as negative control.

To normalize the efficiency of transfection of the plasmid, 2 μg of pTKCAT (a vector with the TK enhancer/promoter fused to the coding sequence of CAT) was cotransfected with pRSV/CREB, pRC/RSV, or pGEM-3 as internal control for normalization. The levels of CAT activity in the control groups (i.e., Figure 1, cells transfected with 2 μg of pTKCAT but without the cotransfection with either pRSV/CREB or pRC/RSV) were used as 100% transfection efficiency. The levels of CAT activity in other groups (i.e., cells cotransfected with 2 μg of pTKCAT and pRC/RSV (at various concentrations) or pRSV/CREB (at various concentrations) were compared with the control group as percentage of transfection efficiency. Subsequently, the IR-hGH levels in groups cotransfected with either pRSV/CREB or pRC/RSV were normalized with the percentage of transfection efficiency as compared with controls (group 1). In each experiment, an additional group of cells was transfected with 2 μg of pRSV CAT (a plasmid with the RSV enhancer/promoter fused to the coding sequence of CAT) as the positive control for the comparison of transfection efficiency. The transfection efficiency of pTKCAT ranged from 25 to 55% compared with pRSCAT.

The depleted FBS was prepared by incubation with 1% activated charcoal and 1% AG 1×8 ion-exchange resin (Bio-Rad Laboratories, Richmond, CA) for 16 h or more at room temperature as described by Samuels et al. (23). This procedure removed endogenous steroid and thyroid hormones from the FBS as demonstrated by Samuels et al. (23).

Effect of Propranolol or Rp-cAMP on the Expression of pOOGH (ANG N-1498/+18) in OK 27 Cells Transfected with pRSV/CREB in the Presence of Isoproterenol

OK 27 cells were plated at a density of 1 to 2×10^5 cells/well in 6-well plates and incubated overnight in DMEM containing 10% FBS. Then, 2 μg of pTKCAT and 5 μg of pRSV/CREB or pRC/RSV were transiently cotransfected into OK 27 cells. After an overnight incubation, the media were replaced with media containing 1% depleted FBS, 10^-9 M isoproterenol, and 10^-6 M propranolol or 10^-4 M Rp-cAMP. After 24 h of incubation, media and cells were collected and kept at -20°C until assay.

Effect of pRSV/CREB on the Expression of pTKGH in OK 13 Cells

OK 13 cells were plated at a density of 1 to 2×10^5 cells/well in 6-well plates and incubated overnight in DMEM containing 10% FBS. Then, 2 μg of pTKCAT with or without 1 to 10 μg of pRSV/CREB per well were transiently cotransfected into OK 13 cells. After an overnight incubation, the media were replaced with fresh media containing 1% depleted FBS and incubated for an additional 24 h. After incubation, cultured media and cells were collected and kept at -20°C until assayed.

Statistical Analyses

The experiments were performed at least three to four times in triplicate. The data were analyzed with t test or ANOVA. A probability level of P ≤ 0.05 was regarded as significant.

Results

Effect of pRSV/CREB on the Expression of pOOGH (ANG N-1498/+18) in OK 27 Cells

Figure 1 shows that the transfection of pRSV/CREB (1 to 10 μg DNA) per well stimulates the expression of pOOGH (ANG N-1498/+18) in OK 27 cells. A dose-dependent relationship between pRSV/CREB and the stimulation of expression of the pOOGH (ANG N-1498/+18) was observed for pRSV/CREB at 1 to 5 μg of DNA. It appears that the maximal stimulation was found with 5 μg of pRSV/CREB. At doses greater than 5 μg of pRSV/CREB, the expression of pOOGH (ANG N-1498/+18) diminished. No significant stimulation of expression of the pOOGH (ANG N-1498/+18) was observed with the control plasmid pRC/RSV (1 to 10 μg). These studies indicate that the expression of pOOGH (ANG N-1498/+18) could be stimulated directly by CREB alone.

Dose-Response Curve of Isoproterenol or Forskolin on the Expression of pOOGH (ANG N-1498/+18) Transfected with pRSV/CREB or pRC/RSV

Figure 2 shows that the addition of isoproterenol (10^-11 to 10^-5 M) stimulated the expression of the pOOGH (ANG N-1498/+18) when transiently transfected with 5 μg of pRSV/CREB in a dose-dependent manner. It appears that the maximal effect of isoproterenol was found with 10^-11 to 10^-9 M isoproterenol. At concentrations greater than 10^-9 M, the enhancing effect of isoproterenol was diminished.

Similarly, Figure 3 shows that addition of forskolin (10^-11
Figure 1. Basal expression of the pOGH (ANG N-1498/+18) in OK 27 cells when transiently transfected with different concentrations of plasmid containing the cDNA for the 43-kD CREB (pRSV/CREB) or with the control plasmid pRC/RSV. The levels of transcriptional activity of pOGH (ANG N-1498/+18) were quantified by the amount of immunoreactive human growth hormone (IR-hGH) in the medium assayed by a specific RIA for hGH (RIA-hGH). The concentration of IR-hGH in the medium of cells (2 x 10^5 cells per well) without transfection with pRSV/CREB or pRC/RSV is considered as the control level. Results are expressed as the mean ± SD of three determinations (triplicates). Solid bars, cells transfected with pRSV/CREB; stippled bars, cells transfected with pRC/RSV. Similar results were obtained from four independent experiments. The data were normalized with the DNA transfection efficiency by cotransfection with 2 µg of pTKCAT as internal controls. The transfection efficiency of pTKCAT in this experiment was approximately 40% compared with the positive control (i.e., cells transfected with 2 µg of pRSVCAT. *P  0.05; **P  0.01; ***P  0.005.

Effect of Propranolol or Rp-cAMP on the Expression of pOGH (ANG N-1498/+18) in OK 27 Cells Transfected with pRSV/CREB in the Presence of Isoproterenol

Figure 5 shows that addition of propranolol (10^-5 M) or Rp-cAMP (10^-4 M) blocked the stimulatory effect of isoproterenol (10^-7 M) on the expression of pOGH (ANG N-1498/+18) in OK 27 cells when transiently transfected with pRSV/CREB.

Effect of pRSV/CREB on the Expression of pTKGH in OK 13 Cells

In OK 13 cells, transfection of pRSV/CREB (1 to 10 µg of DNA per well) had no effect on the expression of pTKGH compared with the controls (in the absence of pRSV/CREB) (Figure 6).

Discussion

Eukaryotic gene transcription is regulated by DNA sequence-specific transcription factors that bind to cis-acting enhancer and promoter elements. The CRE, one of the best studies of such an element, consists of a palindromic octanucleotide, TGACGTCA (24). Within the past 7 to 8 yr, at least 20 different CREB have been cloned, including CREB, activating transcription factor family, and cAMP response element modulator family (for review, see reference 25). CREB 341 (341 amino acid residues) and CREB 327 (327 amino acid residues) (they are tentatively designated as 43-kD CREB) are the best characterized CREB. They are coded from the same gene by two alternatively spliced mRNA. The CREB homodimers bind strongly to the symmetrical palindrome but less well to the asymmetrical sequence such as the CRE (TGACGTAA) of the phosphoenolpyruvate carboxyl kinase gene (26) and CRE (TGACTTCA) of the α-subunit of human glycopro-
Figure 2. Dose–response relationship for the addition of isoproterenol on the expression of pOGH (ANG N-1498/+18) in OK 27 cells cotransfected with pRSV/CREB. Five micrograms of pRSV/CREB or pRC/RSV per well (1 × 10^6 cells) were used in the experiment. The effect of isoproterenol is compared with the control cells (transfected with 5 μg of pRSV/CREB but without the addition of isoproterenol). Results are expressed as the mean ± SD of a minimum of three determinations. Similar results were obtained in four independent experiments. The DNA transfection efficiency for this experiment was approximately 25% compared with pRSV/CAT. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.005.

Figure 3. Dose–response relationship for the addition of forskolin on the expression of pOGH (ANG N-1498/+18) in OK 27 cells cotransfected with pRSV/CREB. Five micrograms of pRSV/CREB or pRC/RSV per well (2 × 10^5 cells) were used in the experiment. The effect of forskolin is compared with the control cells (transfected with 5 μg of pRSV/CREB but without the addition of forskolin). Results are expressed as the mean ± SD of a minimum of three determinations. Similar results were obtained in three independent experiments. The DNA transfection efficiency for this experiment was approximately 35% compared with pRSV/CAT. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.005.

Thus, the symmetry of the CRE sequence and its 5′- and 3′-flanking sequences determine the relative binding affinity and specificity of the CREB.

Our previous studies (19) on the DNA structure of the 5′-flanking sequence of the rat ANG gene showed that the DNA sequence of nucleotides N-795 to N-788 (TGACGTAC) is almost identical to the CRE (TGACGTCATG) of the somatostatin gene (24), except that the last two nucleotides are in reverse order. With such homology, we raised the question of whether the sequence ANG N-795 to N-788 could be a putative
Figure 4. Effect of isoproterenol (10^{-9} M) on the expression of pOGH (ANG N-1498/+18) in OK 27 cells with or without the transfection of pRSV/CREB or pGEM-3. The concentration of IR-hGH in the medium of cells (1 \times 10^5 cells) transfected with 5 \mu g of pGEM-3 or pRSV/CREB, but without the addition of isoproterenol (10^{-9} M), is considered as the control level. Results are expressed as the mean ± SD of a minimum of three determinations. Similar results were obtained from four additional experiments. The DNA transfection efficiency in this experiment was approximately 30% compared with pRSVCAT. *P \leq 0.05; **P \leq 0.01; ***P \leq 0.005.

Figure 5. Inhibitory effect of propranolol or (R)-p-adenosine 3'5'-cyclic monophospho-orthioate (Rp-cAMP) on the expression of pOGH (ANG N-1498/+18) in OK 27 cells when transfected with pRSV/CREB and stimulated by isoproterenol (10^{-9} M). Cells (1 \times 10^5 cells per well) were incubated for up to 24 h in the presence of isoproterenol (10^{-7} M) with or without propranolol (10^{-5} M) or Rp-cAMP (10^{-5} M). The concentration of IR-hGH in the medium of cells (1 \times 10^5 cells) transfected with 5 \mu g of pRSV/CREB, but without the addition of isoproterenol or propranolol or Rp-cAMP, is considered as the control level. The inhibitory effect of propranolol or Rp-cAMP was compared with cells that were incubated with 10^{-9} M isoproterenol. Results are expressed as the mean ± SD of a minimum of three determinations. Propranolol (10^{-5} M) and Rp-cAMP (10^{-5} M) consistently inhibited the stimulatory effect of isoproterenol in three experiments. The DNA transfection efficiency in this experiment was approximately 55% compared with pRSVCAT. *P \leq 0.05; ***P \leq 0.005.
The levels of transcriptional activity of pTKGH were quantified by determinations. Similar results were obtained from three additional transfected with different concentrations of the plasmid pRSV/CREB. The concentration of IR-hGH in the medium assayed by RIA-hGH. The concentration of IR-hGH in the medium of cells (1 × 10^5 cells) without transfection with pRSV/CREB is considered as the control level. Results are expressed as the mean ± SD of a minimum of three determinations. Similar results were obtained from three additional experiments. The DNA transfection efficiency in this experiment was approximately 35% compared with pRSVCAT.

CRE. Indeed, we have demonstrated that forskolin or 8-Br-cAMP directly stimulated the expression of pTKCAT (ANG N-814 to N-761) (a fusion gene containing the 5′-flanking region [nucleotides N-814 to N-761] of the rat ANG gene upstream of the TK promoter fused to a CAT gene in OK cells) (8). Thus, we speculate that CREB might mediate the effect of forskolin or 8-Br-cAMP on the expression of the ANG gene in OK cells via the binding to the putative CRE.

Our present gene transfection experiments showed that pRSV-CREB directly stimulated the expression of pOGH (ANG N-1498/+18) in OK 27 cells (Figure 1). The maximal stimulation was at 5 μg of DNA. At higher concentrations of DNA (i.e., >5 μg), the effect of pRSV/CREB was diminished. At present, we do not know the reasons for this observation. One possible explanation may be that large amounts of DNA transfected into OK cells might exhaust the limited amount of cellular transcriptional factors. Nevertheless, more studies are warranted to clarify this observation.

Isoproterenol and forskolin are known to increase intracellular cAMP levels. Indeed, our previous studies have shown that the addition of isoproterenol increased the intracellular levels of cAMP in OK 27 cells (9). The present studies (Figures 2, 3, and 4) showed that addition of isoproterenol or forskolin further enhanced the stimulatory effect of pRSV/CREB compared with those without the transfection with pRSV/CREB. These studies support the hypothesis that isoproterenol stimulates the synthesis of intracellular cAMP. The elevated intracellular cAMP then activates the cAMP-dependent PKA and phosphorylates the nuclear CREB. Subsequently, the CREB binds to the CRE of the rat ANG gene and enhances the gene expression.

At present, we do not understand why forskolin at 10^{-5} M has an inhibitory effect on the expression of the pOGH (ANG N-1498/+18) in OK 27 cells when transfected with pRSV/CREB. One possible explanation may be that the high concentration of forskolin may exert other physiological effects (i.e., Ca^{2+} transport) and subsequently alter the effect of CREB on the expression of the ANG gene in OK cells. Indeed, more experiments are required to clarify this observation.

Our results also showed that the addition of Rp-cAMP or propranolol blocked the enhancing effect of isoproterenol on the stimulatory effect of pRSV/CREB on pOGH (ANG N-1498/+18) gene expression in OK 27 cells (Figure 5). These studies suggest strongly that the effects of cAMP-dependent PKA and β-adrenergic receptor are involved in the expression of the ANG gene and probably are mediated via the CREB. Indeed, experiments are under way in our laboratory to investigate the direct involvement of cAMP-dependent PKA with the 43 kD CREB (i.e., phosphorylation of 43-kD CREB).

Finally, it appears that pRSV/CREB had no effect on the expression of pTKGH in OK 13 cells (Figure 6). Because the expression of pTKGH in OK 13 cells is driven by the promoter/enhancer DNA sequence of the TK gene, these studies demonstrated that the promoter/enhancer DNA sequence of TK gene is not responsive to the addition of 43-kD CREB. The effect of CREB in OK 27 cells may be mediated via the interaction of CREB with the putative CRE (ANG N-795 to N-788) in the 5′-flanking region of the rat ANG gene of the fusion gene. Indeed, preliminary studies in our laboratory (28) have shown that the CREB is able to bind to the CRE of the ANG gene. Nevertheless, more experiments are under way in our laboratory to confirm this observation.

In summary, our present studies demonstrate that CREB directly stimulates the expression of pOGH (ANG N-1498/+18) in OK 27 cells. The stimulatory effect of CREB could be further enhanced by the presence of isoproterenol or forskolin. The addition of Rp-cAMP or propranolol could block the enhancing effect of isoproterenol. Our studies raise the possibility that the molecular mechanism(s) of the effect of CREB on the renal ANG gene is probably mediated via the PKA and nuclear 43-kD CREB. The local formation of renal Ang II might then modulate the sodium and fluid reabsorption by the renal proximal tubular cells. Hence, the local intrarenal renin-angiotensin system might play a significant role in the modulation of sodium reabsorption.

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