Cyclic AMP Mediated Gene Expression in Bovine Corneal Endothelial Cells

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Purpose. Agents that increase intracellular levels of cAMP mediate gene expression associated with cellular morphology, growth, and/or differentiation via the cAMP response element (CRE). The cAMP element binding protein (CREB) is a transcriptional activator that binds and stimulates gene expression from the CRE in the promoters of cAMP responsive genes. This study was designed to characterize the cyclic AMP (cAMP) transcription apparatus in bovine corneal endothelial cells (BCE).

Methods. CRE transcriptional activity was determined by transient transfection assays using the CRE-chloramphenicol acetyl transferase gene (CRE-CAT) fusion reporter construct. Western blot analyses were performed to determine whether CREB was present in BCE. Mobility shift DNA-binding assay using gel electrophoresis and DNase I protection assays were performed to exclude the possibility of other CRE-binding factors.

Results. The authors identified the transcription factor, CREB, in nuclear extracts from BCE by Western blot analysis and showed that its DNA-binding characteristics are identical to the previously characterized CREB protein by DNase I protection and mobility shift DNA-binding studies. Transient transfection studies using the CRE-CAT reporter constructs revealed that the β-adrenergic receptor agonist, isoproterenol, stimulates gene expression to levels similar to those induced by forskolin, a direct activator of adenylate cyclase (6.0- and 7.2-fold, respectively).

Conclusions. The results suggest that agents that modulate receptors coupled to adenylate cyclase may effect the corneal endothelium by altering gene expression through the second messenger, cAMP. Invest Ophthalmol Vis Sci 1993; 34:2970-2975.

In the anterior chamber of the eye, a variety of extracellular signals, including neuropeptides, prostaglandins, and neurotransmitters have been shown to regulate cellular activity through receptors that are coupled to adenylate cyclase, an enzyme that converts ATP to cyclic AMP (cAMP). The cellular actions of cAMP are mediated through the cAMP dependent protein kinase (A-kinase) holoenzyme, which dissociates into its regulatory (inactive) or catalytic (active) subunits.1 The catalytic subunit then phosphorylates many diverse proteins, such as myosin light chain, to regulate cellular shape or cAMP binding protein (CREB) (see below) to alter patterns of gene expression that may regulate cellular migration, proliferation, or differentiation. The function of cAMP in corneal endothelium is not fully understood, although roles for the maintenance of the normal polygonal shape2 and for stimulating mitosis have been reported.3

The paradigm for cAMP mediated gene transcription has been elucidated and involves the binding of the transcriptional activator, CREB, to the cAMP response element, CRE (Fig. 1).4-7 CREB is a 43-kd nuclear protein that is phosphorylated and activated by the catalytic subunit of A-kinase in response to increased intracellular levels of cAMP. CREB appears to be bound constitutively to its DNA recognition sequence, the CRE, located in the promoter regions of cAMP-regulated genes. The consensus CRE consists of a 5'-TGACGTCA-3' motif, which is the minimal region necessary to confer CAMP responsiveness. The effect of cAMP on gene transcription is rapid. The
expression of c-fos, for example, which contains three such CREs in its promoter, peaks within 60 minutes after cellular stimulation with forskolin, a direct activator of adenylate cyclase.8

The purpose of this study was to characterize the cAMP transcriptional apparatus. We show that agents that alter intracellular levels of cAMP in BCE cells modulate gene transcription through the CRE. Additionally, we show that the transcriptional activator protein responsible for this effect, CREB, is present in these cells and displays DNA-binding characteristics identical to the previously characterized CREB protein.

MATERIALS AND METHODS

Cell Culture

Bovine corneal endothelial cells were established in culture as previously described9,10 from fresh bovine eyes obtained from a slaughterhouse. All procedures adhered to the ARVO statement on the use of animals in ophthalmic and vision research. Cultures were maintained in a 1:1 mixture of Ham’s F12 and TC199 media supplemented with glutamine (3 mg/ml), penicillin (100 U/ml), streptomycin (100 U/ml), and 10% fetal calf serum.

Transfections and Constructs

CRE transcriptional activity was determined by transient transfection assays. BCE cells (approximately 10^6 cells per 100 mm plate) were transfected by calcium phosphate precipitation followed by glycerol shock using 10 μg of CRE-chloramphenicol acetyl transferase (CRE-CAT) fusion reporter construct and 4 μg of RSV β-galactosidase plasmid. The CRE-reporter construct consisted of 71 base pair (bp) upstream of the transcriptional start site of the somatostatin promoter fused ‘5’ to the CAT gene.4 This construct contains one perfect palindromic consensus CRE site and has been widely used to assess cAMP mediated transcriptional activity. Following transfection, the cells were then treated for 36–48 hours with forskolin (10 μM), isoproterenol (5 μM) or ethanol vehicle (control). Cell lysates were prepared and assayed for CAT activity after normalizing for β-galactosidase activity as has been previously described.11 CAT activity was analyzed by thin-layer chromatography and quantified by excision of spots and scintillation counting.

Identifying CREB Activity in BCE Cells

BCE nuclear extracts for Western blot and DNA-binding studies were prepared as described by Dignam and associates.12

Western blot analysis: 25 μg nuclear extract was dissolved in standard 2× loading buffer with 2-mercaptoethanol, denatured, and electrophoresed in a 10% polyacrylamide-SDS gel with a 5% stacking gel. Proteins were then transferred to nitrocellulose. The primary antibody, W39 CREB antiserum (1:250 dilution), was added for 1 hour.13 Staining was performed using an Immun-Blot Alkaline Phosphatase Kit (Bio-Rad). Briefly, this entailed adding a second antibody, goat anti-rabbit IgG alkaline phosphatase conjugate (1:3000 final dilution), for 1 hour followed by chromogenic reaction with BCIT (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt) and NBT (p-nitro blue tetrazolium chloride) substrates.

Mobility shift DNA-binding assay using gel electrophoresis: 5 μg nuclear extract was added to 32P 5’ end-labeled double stranded CRE oligonucleotide probe (3.0 ng) in buffer A (3% polyvinyl alcohol, 12.5 mM Tris, pH 7.9, 6 mM MgCl2, 5% glycerol, 0.05% nonidet P40, 50 mM KCl). Under these conditions, the DNA probe was in vast excess of CREB protein. Fold molar excess of specific (CRE) and nonspecific (AP-1), nonradiolabeled competitor were coincubated at various concentrations, as indicated above each lane. After incubating samples on ice for 10 minutes, protein-DNA complexes were resolved on nondenaturing 6% polyacrylamide gels containing 50 mM Tris, pH 8.3, 2 mM EDTA (pH 8.5). Gels were run at 10 V/cm, dried, and visualized by autoradiography.

DNase I protection assay: 10 μg BCE nuclear extract was incubated with 10 fM 32P-labeled DNA probe, along with 1 μg (dl-dc) competitor DNA (to compete for nonspecific, DNA-binding proteins) in the same buffer as in gel mobility shift assay. Samples were then warmed to 25°C and diluted in an equal volume of 10 mM MgCl2, 10 mM CaCl2. Freshly diluted DNase I was...
added and reactions were allowed to proceed for 1 minute. DNase I digestion was terminated by the addition of 90 μl 20 mM EDTA, 1% SDS, 0.2 M NaCl, 25 mg transfer RNA. Samples were deproteinized by phenol-chloroform extraction and then ethanol precipitated. The products were resolved on an 8% urea-polyacrylamide gel. The DNA probe consisted of T4 polynucleotide kinase labeled HindIII-AvaI 140 bp fragment from the somatostatin promoter.

RESULTS

BCE Cell Response to the β-Adrenergic Receptor Agonist Isoproterenol

Figure 2 shows the effects of treatment of BCE cells with the β-adrenergic receptor agonist (isoproterenol), a direct activator of adenylate cyclase (forskolin), or control (ethanol vehicle) on transcription of the CRE-driven CAT gene. Figure 2a represents an autoradiograph depicting the conversion of [14C] chloramphenicol to acetylated forms as a result of the activity of the enzyme CAT. The acetylated forms are therefore a measure of cAMP mediated transcriptional activity. The acetylated forms were counted separately from the unacetylated forms to determine the percent conversion (acetylated counts/total counts) for each reaction. The ethanol vehicle treated control demonstrates the normal basal transcriptional activity, whereas treatment of BCE cells with either isoproterenol or forskolin results in a dramatic increase in transcriptional activity.

Relative activities were then determined by normalizing to the forskolin-treated cells, which represent maximal activity (100%). Figure 2b shows the mean ± SD of the relative activities for three assays (each assay performed in duplicate). Fold induction in transcriptional activity of the treated groups over the control are shown in brackets above the error bars.

BCE Cells and the cAMP Responsive Transcription Factor CREB

The transcription factor CREB has been reported to mediate the transcriptional response to cAMP in rat brain and in the pheochromocytoma cell line PC-12. To determine if CREB could be mediating the gene expression in BCE cells in response to β-adrenergic receptor stimulation, we identified the protein by Western blot analysis in nuclear extracts of BCE cells. As can be seen in Figure 3a, with the use of CREB antiserum, the Western blot analysis revealed a single 43-kd band representing CREB protein. Western blot analysis demonstrated that CREB protein was present in BCE nuclei. However, to exclude the possibility of other CRE-binding factors, binding characteristics of this BCE CREB were compared to the previously cloned CREB protein by gel retardation and DNase I protection assays. Figure 3b is a mobility shift DNA-binding assay displaying various protein-DNA complexes. The last lane represents recombinant CREB. The faster migrating complex is a degradation product of CREB retaining its DNA-binding capability. Lane 1 is BCE nuclear extract with no competitor DNA. Lanes 2 to 7 use specific (CRE) and nonspecific (AP-1) oligonucleotide competitors at 6.25- to 25-fold molar excess to discriminate between CRE-binding and nonspecific DNA-binding activities. The AP-1 (TPA response element) oligonucleotide was chosen because of its similarity to

FIGURE 2. Forskolin and isoproterenol induction of transcription in BCE cells. (a) Transient transfection of BCE cells with the CRE-CAT reporter, followed by treatment with vehicle (contr), forskolin (forsk), or isoproterenol (isop). Values below represent mean % conversion of [14C] chloramphenicol (the bottom spot) to acetylated forms (the top two spots) of [14C] chloramphenicol. Arrow depicts site of sample loading onto thin layer chromatography. (b) Bar graph representing relative activities (RA) of control and treated groups. Error bars indicate standard deviations. Fold induction in transcriptional activity of the treated groups over the control are shown in brackets above the error bars.
the CRE (one base pair difference) and because it mediates transcriptional responses in the diacylglycerol/protein kinase-C pathway. Lanes 2 to 4 demonstrate the competition of one protein-DNA complex with the CRE oligonucleotide, whereas competition with the AP-1 oligonucleotide has no effect. Furthermore, the complex that specifically competed with the CRE oligonucleotide comigrates with the complex formed by the purified recombinant protein. Figure 3c displays a DNase I protection assay demonstrating that only the CRE portion of the somatostatin promoter is protected from DNase I digestion when BCE nuclear extract is added to the reaction (lane 2). Lane 1, no protein control, shows that the DNA sequence is randomly cleaved by DNase I, even at the CRE site. Lane 3 represents the protection pattern with recombinant CREB protein.

DISCUSSION

The second messenger, cAMP, is known to regulate a diverse range of physiological processes, including cellular shape, differentiation, and proliferation in a variety of cell types including corneal endothelium. The normal polygonal morphology of corneal endothelium is regulated by cAMP via the adenylate cyclase coupled PGE2 receptor. Furthermore, agents that increase intracellular levels of cAMP (forskolin, dibuterol cAMP, 8-bromo cAMP, and cholera toxin) antagonize the loss of polygonal shape and elongation associated with indomethacin treatment, a PGE2 synthesis inhibitor. Similar results have been observed in microvascular endothelium where cAMP deprivation causes a change in cell shape from a polygonal to spindle cell type. Interestingly, this change in morphology was irreversible, implying that the cells had reprogrammed their pattern of gene expression.

Much knowledge has been gained about the intracellular signals that link cAMP activation with gene transcription. In eukaryotic cells, all known effects of cAMP are mediated through the catalytic subunit of A-kinase. A-kinase phosphorylates many cellular substrates, including the transcription factor, CREB. CREB, in turn, is believed to stimulate the expression of a variety of cAMP regulated genes, including c-fos, TGFβ2, TGFβ3, fibronectin, and collagen.
type IV, all of which are important in proliferation, wound healing, and differentiation.22-26

In this report, we examined the molecular mechanisms responsible for mediating the effects of pharmacologic agents, which act through the cAMP pathway in many cell types including corneal endothelium.27-32 We show that β-adrenergic receptor stimulation with isoproterenol and activation of adenylate cyclase with forskolin induces gene expression in BCE cells. As shown by our CAT assays, these changes in gene expression occur at the transcriptional level via the cAMP responsive promoter element, the CRE. Interestingly, β-adrenergic receptor mediated transcriptional activity approximated forskolin-induced activity, suggesting that adenylate cyclase-coupled receptors may function similarly to regulate gene expression.

Several CRE-binding nuclear factors, including CRE-BP1, HB16, and ATF-1 to ATF-8, have been described from a variety of cell types.33-35 To determine the CRE-binding protein responsible for the transcriptional activity, we looked for the presence of the best characterized of these proteins, CREB, by Western blot analysis. These studies clearly demonstrate the presence of the transcription factor CREB in BCE cells. Additionally, DNA-binding studies reveal that BCE CREB binds to the CAMP responsive promoter indistinguishably from the previously characterized CREB protein. Because we could not detect other CRE-binding factors in BCE cells, we hypothesize that CREB may be a key mediator in the maintenance of normal corneal endothelial shape.

In addition to serving a critical role in cellular morphology, cAMP has been associated with proliferation and migration of corneal endothelial cells in vitro.27,38 Because these processes are important after corneal endothelial injury, our results suggest that CREB may also be involved in wound healing through the expression of cAMP regulated genes. CREB may function in corneal wound healing by directly activating the transcription of genes, as in the case of cAMP mediated induction of c-fos.5,36 Conversely, as depicted in Figure 4, CREB may act indirectly by stimulating the expression of transcriptional activators.37

In summary, we have characterized the transcriptional response to stimulation of the cAMP pathway in BCE cells. We have shown that β-adrenergic receptor stimulation modulates gene expression by way of the CAMP response promoter element, the CRE, and the transcription factor CREB. Further studies are under way to elucidate CREB's role in the important processes of cell growth, differentiation, and wound healing in corneal endothelial cells.

Key Words
beta adrenergic receptor agonist, cyclic AMP, transcription, corneal endothelium

References
6. Yamamoto KK, Gonzalez GA, Biggs WH, Montminy MR. Phosphorylation-induced binding and transcri-
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