Expression of cone photoreceptor cGMP-phosphodiesterase α’ subunit in Chinese hamster ovary, 293 human embryonic kidney, and Y79 retinoblastoma cells

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Purpose: A functional protein is required for structure/function analysis of cone photoreceptor cGMP-phosphodiesterase α’ subunit (PDEα’). The purpose of this study was to express enzymatically active PDEα’.

Methods: Three expression vectors were constructed for transient and stable expression of PDEα’: pC57 (transient) was obtained by subcloning bovine PDEα’ cDNA into the pCIS2 expression vector; pNC57 (stable) was constructed by inserting the neo gene controlled by the mouse phosphoglcerate kinase-1 gene promoter into the pC57 vector; and pFC57 (transient) was generated by fusing the sequence encoding the FLAG peptide to the 5’ end of the coding region of PDEα’ cDNA. The recombinant plasmid DNAs were introduced into HEK293, CHO, or Y79 retinoblastoma cells using the calcium phosphate-mediated transfection procedure or lipofectamin. Northern and western blot hybridizations were used for RNA and protein analysis, respectively.

Results: Northern blots of both HEK293- and CHO-transfected cells showed strong expression of a 3 kb transcript corresponding to PDEα’. cGMP-PDE activity measured in homogenates of transiently and stably transfected cells ranged between 1.5 and 2.2 nmol cGMP hydrolyzed/min x mg total protein, a level of PDE activity slightly greater than that previously reported for the individual rod-photoreceptor PDE subunits transiently-expressed in HEK293 cells. Western blots of these cell homogenates showed a low level of expressed PDEα’. Transfection of Y79 retinoblastoma cells, that have been shown to express rod and cone PDEs endogenously, with the construct containing cone PDEα’ cDNA fused to the FLAG peptide resulted in a protein with no enzymatic activity.

Conclusions: Our results demonstrate that both HEK293 and CHO cells are capable of expressing functionally active cone PDEα’. High level of mRNA transcription and relatively low protein synthesis efficiency indicates the presence of a post-transcriptional control mechanism regulating overall expression of PDEα’ in HEK293 and CHO cells.

The vertebrate retina is composed of rod and cone photoreceptor cells that have homologous proteins involved in phototransduction cascades, but differ in their signal response characteristics. Rod photoreceptors are 100-fold more sensitive to light stimulation than cones, but cone photoreceptors have a faster response/recovery time and can adapt to a greater range of light intensities than rods [1-3]. The final step of signal amplification in both rod and cone cascades is the hydrolysis of guanosine-3’,5’-cyclic monophosphate (cGMP) by a cell-specific cGMP-phosphodiesterase (cGMP-PDE).

Rod cGMP-PDE consists of an α subunit (PDEα, 88 kDa) and a β subunit (PDEβ, 84 kDa) that comprise the catalytic core of the enzyme, two γ subunits (PDEγ, 11 kDa) that inhibit the hydrolytic activity of PDEαβ [4,5], and a δ subunit that is capable of solubilizing the rod-membrane bound enzyme [6]. The cone enzyme has a similar structure with a homodimeric catalytic core composed of two α’ subunits (PDEα’, 93 kDa) and possibly two or three low molecular weight (13-15 kDa) subunits [7]. Although both enzymes have similar Vmax values when purified [7], they differ in apparent affinities for their respective inhibitory subunits [8]. This may be the result of specific differences at the amino acid level, since there is approximately a 77-82% homology between the PDEα, PDEβ, and PDEα’ subunits.

Site-directed mutagenesis can be used to study the role of individual amino acids in protein-protein interaction sites, ligand binding sites, and catalytic moieties. Such functional studies have been carried out with the rod PDE γ subunit [9], but not with the catalytic subunits of the enzyme, probably because of the difficulty in obtaining sufficient amounts of the native proteins. It would be extremely beneficial to get adequate quantities of these enzymatically active proteins to carry out experiments aimed at obtaining a better understanding of their role in normal development, differentiation, and maintenance of the photoreceptor cells in the retina. This information would also explain how certain mutations in the corresponding genes could cause retinitis pigmentosa and congenital stationary night blindness [10-15]. We have expressed both PDE α and β subunits by transient transfection in HEK293 cells [16]. This mammalian expression system allowed us to obtain proteins with 20-40-fold greater enzymatic activities than other systems utilizing E. coli and Sf9 insect cells [17,18], but the cGMP-PDE activity measured for each PDE subunit was 40-50-fold lower than that in adult wild type C57Bl/6J mouse retina (90-110 nmol cGMP hydrolyzed/min x mg total...
protein). This may be due to reduced stability and subsequent proteolytic degradation of the individually expressed rod PDE subunits or to a change in conformation upon assembly of a homodimeric (αα or ββ) catalytic core (which has been shown to occur in the rd1 mouse [19]).

In this study, we undertook the expression of the cone photoreceptor PDEα' subunit in HEK293, Chinese hamster ovary cells (CHO), and Y79 retinoblastoma cells with the goal of finding an appropriate cell culture system that would provide a reasonable amount of enzymatically active protein for structure/function studies. In contrast to the rod PDE subunits, we expected that the cone enzyme would be assembled as a functional homodimeric catalytic core and thus exhibit characteristics particular to the native protein. Our results demonstrate that both HEK293 and CHO cells can be used to express functionally active PDEα' and also provide evidence for the presence of a post-transcriptional regulation mechanism that controls the overall expression of the enzyme.

METHODS

Vector construction: Vector pc57 for transient expression of the cone photoreceptor PDEα' subunit (Figure 1) was obtained by subcloning a cDNA fragment containing the complete coding region and 3' untranslated region (UTR) of PDEα' as well as 50 bp of its 5' UTR, between the Xba I and Xho I sites of the pcIS2 vector (Genentech, San Francisco, CA). For stable expression of cone PDEα', the pcNC57 vector was constructed as follows: First the fragment containing the mouse phosphoglycerate kinase-1 (PGK-1) promoter and the neo gene was released from the pPNT plasmid [20] using Xho I and Xba I and blunted. This fragment was then subcloned into the blunted Sal I site of pc57.

The pcFC57 vector was constructed to express PDEα’ in Y79 and Weri retinoblastoma cells. This was achieved by PCR using primers FC1 (CCT CTA GAG ACT ACA AGG A TG ACG A TG ACA AGA TGG GTG AGA TCA GCC AAG AGA TGG AGT ATG ACG ATG ACA AGA TGG GTG AGA TCA GCC AAG AGA CCG TGG AG) and P3 (TAT TTG TAA CCA TTA TAA GCT GCA ATA) and pc57 DNA as a template. FC1 contains a FLAG sequence (synthetic epitope tag encoding 8 amino acids) fused to the PDEα' coding sequence and the site for Xba I and Xho I and blunt-ended. This fragment was then subcloned into the blunt Sal I site of pc57.

RNA isolation and northern blot analysis: Total RNA from cultured mammalian cells was isolated using TRIzol (GibcoBRL). To remove any DNA contamination, the samples were treated with RNase free DNase for 30’ at 37°C. 5 μg of total RNA/lane were separated by agarose gel electrophoresis (1% agarose, 2.2 M formaldehyde) and transferred onto HyBond N+ nylon membranes (Amersham Life Sciences, Inc, Arlington Heights, IL). As a probe for hybridization we used a PDEα' cDNA fragment labeled by the random priming technique [22]. After hybridization, the membranes were washed at a final stringency of 0.2X SSC (0.15 M NaCl/0.015 sodium citrate pH 7.6), 0.1% sodium dodecyl sulfate (SDS) at 58°C and exposed to X-ray film (Amersham Life Sciences, Inc) for 12-24 h at room temperature.

cGMP-PDE assay: Cells were resuspended in homogenization buffer (10 mM Tris, pH 7.6, 1 mM ethyleneglycol tetraaetic acid (EGTA), 0.1 mM phenylmethyl sulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin) at a 1:10 ratio and homogenized in a Duall tissue grinder. Samples were centrifuged in 2.0 ml Eppendorf microfuge tubes at 8000x g for 20 min at 4°C. The supernatant fractions were removed and the protein concentrations were determined by the method of Peterson [23] using bovine serum albumin (BSA) as standard. Aliquots were removed for determination of cGMP-PDE activity as previously described [24] using 100 μM cGMP as substrate. Briefly, each reaction was carried out in triplicate using 50 to 80 μg of protein. In the first step, after incubation for 10 min at 37°C in 40 mM Tris buffer pH 7.6, 5 mM MgCl2, 1 mM DTT and 2x105 cpm 3H-cGMP, the reaction was terminated by heating at 80°C for 3 min. In the second step, the samples were brought to room temperature, calf intestinal alkaline phosphatase (0.4 U/sample) was added, and incubation proceeded at 37°C for 10 min. The reaction was stopped by addition of a 1:6 mixture of AG1-X2 (50-100 mesh, Biorad Laboratories, Hercules, CA) resin to water. The resulting 3H-guanosine was separated from other nucleotides after a 20 min incubation at 4°C. Radioactivity in the supernatant was quantified in a scintillation counter.

Polyacrylamide gel electrophoresis and immunoblots: In order to optimize the resolution of proteins in the 80-100 kDa range, we used amodification of the double-inverted gradient
polyacrylamide gel described by Zardoya et al. [25]. Briefly, the gel consisted of three layers: a stacking gel, 4.5% T/1.5% C (where “%T” and “%C” are the total acrylamide and crosslinking concentrations by convention), a separating gel (9.5% T/1.5% C to 6.5% T/1.5% C), and an inverted gradient resolving gel (9.5% T/1.5% C to 6.5% T/1.5% C). Samples were electrophoresed in a tris/tricine buffer system as described by Schagger and von Jagow [26] for 22 h at 35 mA constant current, then transferred to nitrocellulose by the method of Towbin et al. [27] at 4 V/cm, 10 °C, overnight. Nitrocellulose membranes were blocked in 3% BSA, 0.1% Tween-20 in Tris buffered saline (TBS, 500 mM NaCl, 20 mM Tris, pH 7.6) for 2 h at 37 °C, and blots were then incubated overnight with primary antiserum in the blocking solution supplemented with 0.5% normal goat serum. Blots were visualized with either the Amplified-Alkaline Phosphatase kit (BioRad Laboratories) or the Enhanced Chemiluminescence kit (ECL; Amersham Life Sciences, Inc). The polyclonal antiserum (BC18/50) used in these experiments was generated against an 18-mer peptide (EKM ETE EEA IKY VTI DPT) corresponding to amino acid residues 692 to 709 of the bovine cone PDEα’ subunit. This sequence has about 27% homology with both the rod PDEα and β catalytic subunits. As control, we used a crude bovine cone cGMP-PDE enriched sample obtained after subjecting a ROS extract to ion exchange chromatography on diethylaminoethyl (DEAE) Sephadex resin by the method of Gillespie and Beavo [7]. Proteins containing FLAG were immunoprecipitated using antibodies recognizing that peptide (Stratagene, La Jolla, CA).

RESULTS

Transient and stable transfection of HEK293 and CHO cells and Northern blot analysis: For transient transfection of HEK293 and CHO cells we used the pC57 vector (Figure 1) that contains the full length PDEα’ cDNA vector driven by the human CMV promoter and enhancer region. pCIS-derived plasmids also contain the SV40 origin of replication, and their amplification in mammalian cells can be achieved by the presence of T antigen [28,29]. A mixture of pC57 and pRSVT (T antigen expression vector) plasmid DNAs in a 10:1 ratio was used for transfection to increase efficiency of expression.

In our previous studies on expression of the rod-specific PDEα and β subunits in HEK293 cells, we found that although high levels of their mRNAs were obtained after transfection, small amounts of the corresponding proteins were produced. In order to increase the level of PDEα’ synthesis, we constructed another vector (pNC57) intended to achieve stable instead of transient expression of the cone-specific protein.

CHO and HEK293 cell lines were utilized for transient (using vector pC57) and stable (using vector pNC57) transfection studies to determine if efficiency of expression varied with cell type. Northern blots of total RNA isolated from the transfected cells are shown in Figure 2. As expected, RNA from CHO and HEK293 cells transfected with the pCIS2 vector did not hybridize with the labeled PDEα’ cDNA fragment. A distinct single band of about 3 kb corresponding to the PDEα’ mRNA is present in all three lanes containing total RNA from transiently (lane 3 and 5) or stably (lanes 4) transfected cells. Lane 5 shows more intense signal than lane 3 suggesting that the CMV promoter and enhancer region controlling PDEα’ cDNA expression is more efficient in the HEK293 than in the CHO cell line. This observation is in agreement with previous studies that have shown that the human CMV promoter is very active in adenovirus transformed human cell lines [30].

![Figure 1](http://www.molvis.org/molvis/v9/a12>)

Figure 1. PDEα’ expression constructs. Constructs used for expression of cone cGMP-PDEα’ subunit. CMV: human cytomegalovirus immediate early (IE) enhancer and promoter region. E: exon 1 of the major IE gene. I: intron composed of a 5′ splice sequence from the major IE gene of human CMV attached to a synthetic 3′ splice sequence. F: sequence corresponding to the FLAG peptide. MCS: multiple cloning site. Xbal and XhoI: restriction sites used to subclone the PDEα’ cDNA. SV40 polyA: cleavage/polyadenylation signals derived from SV40. PGK-1: mouse phosphoglycerate kinase-1 promoter; neo: neomycin gene.

![Figure 2](http://www.molvis.org/molvis/v9/a12>)

Figure 2. Northern blot analysis of PDEα’. Northern blot analysis of total RNA (5 µg/lane) isolated from: Lanes 1 and 2: CHO and 293 cells transfected with pCIS2, respectively; lane 3: CHO cells transiently transfected with pC57; lane 4: stably transfected 293 cells with pNC57; lane 5: 293 cells transiently transfected with pC57.
tively higher level of PDEα’ mRNA in lanes 3 and 5 than in lane 4 can be explained by the presence of multiple copies of expression plasmid in the transiently transfected cells.

cGMP-PDE activity and western blot analysis of transiently and stably transfected HEK293 and CHO cells: cGMP-PDE activity in both HEK293 and CHO cells transiently transfected with the pC57 vector (1.5 and 1.4 nmol cGMP hydrolyzed/min x mg total protein, respectively) was about two-fold higher than that measured in cells transfected with the pCIS2 vector alone (Figure 3A,B). The results shown were obtained using cells harvested 48 h after transfection. Similar cGMP-PDE activity was observed in cells that were analyzed 24 or 72 h after transfection. cGMP-PDE activity in these cells is due to the presence of other phosphodiesterases (not photoreceptor-specific) that can use cGMP as substrate [31]. The ROS-1 monoclonal antiserum against cGMP-PDE has been previously shown to specifically inhibit both the rod and cone enzymes [32,33]. When this antiserum (a generous gift from Dr. Richard Hurwitz) was incubated with cell homogenates from pCIS2- and pNC57-transfected CHO cells, it only inhibited the cGMP-PDE activity of the pNC57-transfected CHO cells, indicating the presence in these cells of the cone PDE α’ subunit (Figure 3C).

Proteins from a bovine cone cGMP-PDE enriched fraction and pC57-transfected HEK293 and CHO cells were separated by gel electrophoresis and analyzed on western blots using the Amplified Alkaline Phosphatase protocol. Although similar results were observed with pC57-transfected CHO and HEK293 cells, Figure 4 only shows the results obtained with the pC57-transfected CHO cells and with the bovine cone PDE-enriched sample. In lane 1, the latter was incubated with the αT1 polyclonal peptide antiserum previously characterized [16]. This antiserum was raised against a 17-mer peptide sequence bearing 100% homology between the rod PDE α and β subunits and the cone PDE α’ subunit, denoted as R and C, respectively. Only the higher molecular weight polypeptide C is detected by the BC18/50 polyclonal peptide antiserum against cone PDEα’ (Figure 4, lane 2). This signal could be competed out by the 18-mer peptide (10 µg/ml) against which BC18/50 was raised (Figure 4, lane 4), confirming that the band corresponds to PDEα’. A polypeptide of approximately 200 kDa was determined to be a non-specific cross-reactive product of the enriched cone cGMP-PDE fraction (it was present on a blot incubated with pre-immune serum, Figure 4, lane 6). Several other minor bands were detected in lanes 3 and 5 that contain proteins from pC57-transfected CHO cells.

Figure 3. cGMP-PDE activity. cGMP-PDE activity of extracts from pCIS2- and pC57-transfected HEK293 (A) and CHO (B) cells. The assay was carried out using 100 µM cGMP as substrate at 37 °C and ~80 µg of total protein. Results are from typical experiments conducted in triplicate (±SD). C: Inhibition by the ROS-1 monoclonal antibody cGMP-PDE activity in homogenates from pCIS2- and pNC57-transfected CHO cells. Cell extracts (80 µg protein) were incubated with a 1:20 dilution of ROS-1 antiserum at 4 °C for 3 h, then assayed for cGMP-PDE activity with 50 µM cGMP as substrate. Activities are presented as percent of that of control extracts which were incubated without the ROS-1 antiserum.
These bands were also present in lane 7, that had been incubated with the pre-immune serum only, indicating that they do not correspond to PDEα'. These data show that the cGMP-PDE activity seen in pC57-transfected CHO cells is obtained from cone PDEα' expressed at a level that is beneath the threshold of detection of the western.

Stably transfected HEK293 cells showed an approximate 60% increase in cGMP-PDE activity when compared with the transiently transfected HEK293 cells (2.3 nmol cGMP hydrolyzed/min x mg total protein vs. 1.5 nmol/min x mg total protein). A protein corresponding to PDEα' was detected after reacting homogenates of the stably transfected cells with the BC18/50 antibody on a western blot (Figure 5A, lane 4). This band was competed out by the BC18/50 peptide (Figure 5B, lane 4).

**Transient transfection of Y79 retinoblastoma cells:** Two retinoblastoma cell lines, Y79 and Weri, that express phototransduction proteins [34,35] were also used for expression of the recombinant cone PDEα'. Prior to transfection we measured cGMP-PDE activity in Weri and Y79 cells (11.3 and 3.9 nmol/min x mg protein, respectively) using 250 µM cGMP as substrate. Endogenous expression of PDEα' could complicate the interpretation of the transfection experiments. For this reason, we transfected Weri and Y79 cells with the pFC57 vector (Figure 1) that contains a sequence for the FLAG peptide attached to the 5' end of the PDEα' cDNA coding re-

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**Figure 4.** Western blot analysis of the proteins from pC57-transfected CHO cells. Immunoblots containing proteins from a cone PDEα' enriched fraction (60 µg total protein, lanes 1, 2, 4, and 6) and pC57-transfected CHO cells (80 µg total protein, lanes 3, 5, and 7). Lane 1 was incubated with αT1 peptide antiserum (1:1000 dilution) which recognizes rod α and β (R) and cone (C) cGMP-PDE catalytic subunits. Lanes 2 and 3 were incubated with BC18/50 polyclonal peptide antiserum (1:1000 dilution). Lanes 4 and 5 were incubated with BC18/50 antiserum and the BC18/50 peptide against which the antibody was raised (10 µg/ml). Lanes 6 and 7 were incubated with pre-immune serum from normal rabbit (1:500 dilution). BioRad Laboratories pre-stained molecular weight markers, broad range, were used as standards (Mr): 203 kDa, myosin; 116 kDa, β-galactosidase; and 83 kDa, bovine serum albumin.

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**Figure 5.** PDEα' expression in HEK293 and Y79 cells. Immunoblots containing protein from bovine cone PDEα' enriched fraction (60 µg total protein, lane 1), Weri-Rb cells (110 µg total protein, lane 2), Y-79 cells (110 µg total protein, lane 3), pNC57-stably transfected HEK293 cells (100 µg total protein, lane 4), and pCIS2-transfected HEK293 cells (100 µg total protein, lane 5). A: Immunoblot incubated with BC18/50 polyclonal peptide antiserum (1:1000 dilution). B: Immunoblot incubated with BC18/50 antiserum and competed with the BC18/50 peptide (10 µg/ml). The arrow indicates the position of the immunoreactive polypeptide identified as the cone-photorceptor PDE α' subunit. C: Immunoblot probed with antibodies recognizing FLAG peptide. Proteins from non-transfected and transfected Y79 cells are represented in lanes 1 and 2, respectively. The arrow points to the band corresponding to the FLAG-PDEα'. Molecular weight markers are the same as in Figure 4.
gion. We then compared on western blots the bovine cone PDEα' (from the PDEα' enriched preparation) with that of the Y79 and Weri cells transfected with pFC57, as well as with the stably transfected 293 cells. As a negative control we used the pCIS2-transfected 293 cells. Using the BC18/50 polyclonal peptide antiserum, a single immunoreactive polypeptide corresponding to the PDE α' subunit was detected in all lanes except the control pCIS2-transfected HEK293 cell lane (Figure 5A). The immunoreactivity was abolished with peptide competition with the peptide used to generate the antiserum (Figure 5B), indicating that this protein was responsible for the cGMP-PDE activity measured in the retinoblastoma cell lines. Endogenous expression of PDEα' could complicate the interpretation of the transfection experiments. For this reason, we transfected Weri and Y79 cells with the pFC57 vector (Figure 1) that contains a sequence for the FLAG peptide attached to the 5' end of the PDEα' coding region. The chimeric protein expressed in the transfected retinoblastoma cells had a molecular weight corresponding to that of PDEα' plus the FLAG peptide (Figure 5C). It was immunoprecipitated with antibodies that recognize the FLAG peptide. Unfortunately, FLAG-PDEα' did not have cGMP-PDE activity, most likely due to the distortion of the native PDEα' structure by the FLAG peptide.

**DISCUSSION**

Based on the results of our previous studies on the expression of the α and β subunits of rod-specific cGMP-PDE, we postulated that attempting the expression of PDEα' would be straightforward. The expressed protein would be able to assemble as a stable homodimer, just as it is found in cone photoreceptors.

Two cell lines, HEK293 and CHO, were used for transfection of the PDE α' subunit. The cGMP-PDE specific activity of the expressed cone PDEα' was comparable to those of the rod PDE α and β subunits expressed in HEK293 cells. However, western blots using the Amplified Alkaline Phosphatase protocol failed to detect the PDE α' subunit, in contrast with our ability to detect the individually expressed rod PDE α and β subunits on immunoblots [16]. These results suggested that the cone PDE α' subunit is enzymatically more active than the rod PDE α and β subunits.

The observed difference between PDEα' mRNA and protein levels in transiently transfected cells could be explained by translational or post-translational regulation of PDEα' expression. Although each transfected cell may produce high levels of message due to amplification of the pC57 plasmid, the synthesis of the foreign protein in these cells could be inhibited at the translational level or the synthesized protein could have a very high turnover rate.

To increase the level of PDEα' expression we stably transfected HEK293 cells with the pNC57 vector. We noted higher cGMP-PDE activity in these stably-transfected cells than in pC57 transiently-transfected HEK293 cells, and we were able to detect the expressed polypeptide using the ECL method (Figure 5). However, the level of protein expressed in stably-transfected HEK293 cells was still lower than that of the endogenously expressed PDEα' in Y79 or Weri retinoblastoma cell lines (Figure 5). To test the possibility that these cell lines have a more suitable intracellular environment for efficient expression of a recombinant PDEα', we transiently transfected Y79 cells with the pFC57 construct, but the protein synthesized in these cells had no enzymatic activity. It is possible that the addition to the N-terminal end of PDEα' of five acidic and two basic amino acid residues (FLAG peptide) may affect the correct protein folding and consequently its function.

Our results demonstrate that both HEK293 and CHO cell lines are capable of synthesizing enzymatically active cone PDEα', but they limit the total amount of expressed PDEα' either due to low protein translation or increased protein turnover. Possibly there is need of a co-expressed cone PDEγ or δ subunit to stabilize and correctly fold the catalytic core [36].

**ACKNOWLEDGEMENTS**

Supported by National Institutes of Health grants EY02651 and EY0331, a grant from The Foundation Fighting Blindness, and by an unrestricted grant from Research to Prevent Blindness. DBF is the recipient of a Research to Prevent Blindness Senior Scientific Investigators Award.

**REFERENCES**


