Identification of the expression of inhibitory gamma subunit of phosphodiesterase-6 in non-retinal murine tissues

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Phosphodiesterases (PDEs) are enzymes that maintain the integrity of various cyclic nucleotide signaling pathways by regulating the cellular levels of cyclic nucleotide secondary messengers. PDE-6 is the only member of the phosphodiesterase family that is known to be regulated by a G-protein. The γ-inhibitory subunit of PDE-6 facilitates the interaction between G-protein (transducin) and PDE-6 and PDEγ is the only known molecule via which G-protein and phosphodiesterase interact. We have found the expression of PDEγ-like transcripts and proteins in non-retinal tissues such as testis and lungs by both RT–PCR and Western blotting. We identified two main isoforms of PDEγ, which we name rod PDEγ and cone PDEγ. The cone PDEγ undergoes splicing to give rise to a short isoform, which has a truncated C-terminal. We have also shown that p14 is a mixture of rod and cone gamma, while p18 corresponds to the phosphorylated rod gamma. Our results are suggestive of a widespread physiological interaction between G-protein and phosphodiesterases.

PHOSPHODIESTERASES (PDEs) are a superfamily of enzymes that metabolizes cyclic nucleotide secondary messengers such as cAMP and cGMP. As these secondary messengers are the converging points for a wide variety of cellular signal transductions mediated by hormones, eicosanoids and neurotransmitters, the precise regulation of their intra-cellular levels is essential for the maintenance of physiological functions in body and PDEs are dedicated towards this role. The PDE superfamily consists of 11 main members ranging from PDE-1 to PDE-11. PDE-6, an isoform believed to be confined to retina, is unique as it is the only PDE that is regulated by a G-protein[1]. PDE-6 in rod photoceptors is an $\alpha\beta\gamma_2$ hetero-tetramer with $\alpha\beta$ catalytic subunits and γ-inhibitory subunits. When light strikes on rod photoceptors the photopigment rhodopsin undergoes photoisomerization and then activates the G-protein transducin, which binds to γ-inhibitory subunits of PDE-6 and detaches it from the catalytic subunits. This leads to the activation of PDE-6, resulting in the metabolism of cGMP to 5′GMP. As cGMP keeps the Na^+ and Ca^{2+} channels open leading to the maintenance of the dark state in photoceptors, PDE-6 activation closes these channels and brings about the sense of vision. Majority of the drugs sold world-wide target either G-protein-coupled receptors or its immediate effectors. Manipulating PDE activity resulted in drugs ranging from classical anti-asthmatic Theophyline to block-buster drug Viagra. Therefore, it is intriguing to probe if the interaction between G-proteins and PDEs is more widespread. To test if this interaction is more widespread, an approach will be to check the expression of PDEγ in tissues, that are abundant in other PDE isoforms, as PDEγ is the only known molecule to facilitate the interaction between G-protein and PDE.

The rationale for the hypothesis that PDEγ-like molecules could be expressed in non-retinal tissues in association with other PDE isoforms, stems from the following observations. Among the PDE superfamily, PDE-5 and PDE-10 have striking structural similarity with PDE-6 and it is tempting to probe if PDEγ-like endogeneous inhibitory proteins could inhibit the activity of PDE-5 and PDE-10. All the three isoforms have two N-terminal GAF domains and a C-terminal catalytic domain[2]. The C-terminal catalytic domain of these isoforms undertakes preferential metabolism of cGMP over cAMP in contrast to the catalytic domain of the other isoforms. All the three isoforms have similar inhibitory sensitivities (Zaprinast inhibits all three isoforms, while the so-called PDE-5 specific inhibitor Sildenafil/Viagra is found to inhibit PDE-6 and is found to cause bluish haze in patients)[3]. Apart from this, Lochhead *et al.*[4] have identified two proteins; p14 and p18 in guinea pig airway smooth muscle cell line (rich in PDE-5) upon Western blotting with antibodies raised against the central polycationic region of PDEγ. Further to this, they have demonstrated that recombinant PDEγ inhibits PKA-induced PDE-5 activity[2]. A recent publication has demonstrated that over-expression of PDE6A promoter fragment resulted in the weak expression of PDE6A in murine brain[5]. All these data are tempting enough to probe the expression of PDEγ or PDEγ-like inhibitory peptides in non-retinal tissues. We used testis, lungs and various brain regions from *Mus musculus* for this study as they are rich in PDE-10, PDE-5 and both isoforms respectively[3].

Mice (*Mus musculus*) were sacrificed and tissues were dissected out quickly and stored in – 80°C. The tissues were then ground using a mortar and pestle containing liquid nitrogen. The sample was homogenized using QIA shredder followed by passing through 23-G needle fitted to a syringe. The RNA was extracted from the homogenate using RNeasy kits (Qiagen, UK). A clean-up for DNA contamination was performed using DNAase treatment at 37°C for 1 h.

dNTP, oligo DT [Y01212], DTT [700147], first strand buffer [Y00146], superscript II reverse transcriptase [18064-014], magnesium chloride [Y02016], 10 × PCR buffer [Y02028], Taq DNA polymerase [10342-020],

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cone EST forward primer, cone EST reverse primer, Y forward primer, Y reverse primer, DNAase [18068-015], 10 × DNAase I Rxn buffer [Y02340] and 100 bp DNA ladder [15628-019] were obtained from GIBCO BRL, UK. Ethidium bromide was obtained from Sigma-Aldrich.

The RNA and DNA concentrations were measured using Gene QuantII [80-2105-98], Pharmacia Biotech. A total of two microgram of RNA was used in one reverse transcription reaction, which was primed by 500 ng of oligoDT and catalysed by superscript II reverse transcriptase (450°C for 2 h). Two hundred nanogram of the cDNA obtained was used for the PCR, which was primed with 100 pmol of both forward and reverse primers and catalysed by Taq DNA polymerase. Cone gamma was amplified using cone EST forward primer [5′-CGGGATCCCGCCACCATGAGCGACAGCCCTTGCC-3′] and cone EST reverse primer [5′-CCCAAGCTTGGGTCCTCAGATGATCCCGAACTG-3′], while rod gamma was amplified using Y forward primer [5′-CGGGATCCCGCCACCATGAGCGACA-3′] and Y reverse primer [5′-AAGCTTGGGAAGGCACTCAAGAGCA-3′]. PCR was performed using 'DNA engine' (model: PTC200) MJ research. The thermal cycles used were: 1 cycle (70°C for 10 min); 35 cycles (95°C for 1 min 30 s; 50°C for 30 s; 72°C for 1 min 40 s); 1 cycle: 72°C for 10 min. The reaction mixture was then run on 2% w/v agarose gel and stained with ethidium bromide. The gel was photographed using the 'Electrophoresis Documentation & Analysis System 290', Kodak.

The purified amplicons were sequenced in both directions, on a PE applied Biosystems Division Model 373A automated DNA sequencer using the primer and a Big Dye terminator cycle sequencing kit.

One hundred and fifty microgram of protein was loaded in each well and run for 2 h in 8% acrylymide gel and then electroblotted for 90 min. The membrane was blocked in 1 × PBS + 3% milk powder for 3 h and then probed with anti-PDEγ (rabbit polyclonal) antibody for 90 min, washed with 1 × PBS and then incubated with anti-rabbit secondary antibody for 45 min, followed by 1 h washing with 1 × PBS. Detection of protein bands was done using Chemiluminescence Reagents ECL Western Blotting Analysis System, that is commercially available from Amersham Pharmacia Biotech, UK.

To obtain enriched PDEγ, we immunoprecipitated PDE gamma from crude total cell lysate using beads (Amersham Pharmacia Biotech, UK) coupled to antibody raised against the CPR of PDEγ. The immunoprecipitate obtained was then treated with the serine/threonine-specific phosphatase, PP2A (Upstate Biotechnologies, NY). This was accomplished by incubation with 0.5 units of PP2A in 60 µl of PP2A reaction buffer [20 mM Hepes (pH 7.0), 1 mM dithiothreitol, 7 mM $MgCl_2$, 0.01 mM $MnCl_2$, 100 µg/ml bovine serum albumin, 5 µg/ml aprotinin, 1 µg/ml leupeptin] for 1 h at 37°C.

The rod gamma knock out (homozygous) was primarily generated by the group of Arshavsky VY, Boston.

RT–PCR using EST cone forward primer and EST cone reverse primer gave two bands of 252 and 211 base pairs respectively. The 252 bp band was identified to be cone gamma, while the 211 bp band was identified to be a novel transcript. RT–PCR using Y forward and Y reverse primers was run on a 2% agarose gel (Figure 1). A strong band corresponding to 284 bp was obtained, which was cut out and sequenced using automated sequencing. This 284 bp band was identified as rod gamma.

Similar results were obtained by RT–PCR of total RNA obtained from lungs but more cDNA (about 800 ng) obtained from reverse transcription had to be introduced into the PCR reaction to get bands which have similar intensity to bands obtained from testis (Figure 1). The respective bands obtained using primers for rod and cone gamma from both testis and lungs had identical sequences (Figures 2 and 3). We simulated translation of the nucleotide sequences (courtesy: www.expasy.ch) and found that N-terminal region of the cone gamma and rod gamma varies from one another, while the central polycationic region and the C-terminal regions are identical.

We identified a novel transcript of cone PDEγ, which was amplified by RT–PCR using primers constructed using the EST sequence encoding rat cone PDEγ. This transcript with 211 bp, when compared to cone gamma cDNA is predicted to encode a shorter isoform. The amplicon was not obtained when reverse transcription was omitted from the first strand DNA synthesis step (data not shown). This transcript has a 41 bp deletion, which is predicted to result in a frame change and an early termination to produce a truncated protein, because of an early 'in frame' stop codon. The protein sequence obtained upon translation of the nucleotide sequence of this novel transcript has identical N-terminal and central polycationic region with that of long cone gamma, but the C-terminal region is truncated.

RT–PCR of mRNA from brain using primers for rod and cone gamma failed to generate any amplification products. The results were unchanged upon usage of a more sensitive reverse transcriptase, Sensiscript (Quiagen, UK). But primers for GAPDH housekeeping gene (positive control) gave rise to a specific band of expected size (data not shown). Therefore it appears that PDEγ transcripts are absent in brain.

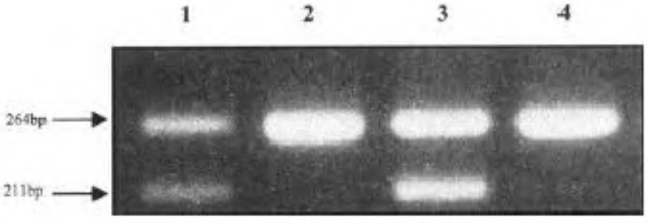

Figure 1. RT–PCR analysis of PDEγ in testis and lungs. Lane 1: Lung cone gamma, Lane 2: Lung rod gamma, Lane 3: Testis cone gamma, Lane 4: Testis rod gamma.

Figure 2. Nucleotide sequences of PDE rod gamma.

Figure 3. Nucleotide sequences of long and short isoforms of PDE cone gamma.

Western blotting studies using antibodies raised against the central polycationic region of PDEγ identified two bands of molecular weight 14 and 18 kDa (Figure 4). This is the first report that identifies p14 and p18 in mice. Earlier these proteins have been identified in guinea pig airway smooth muscle[4]. We found that p18 is completely absent in rod PDEγ knock out mice, while the intensity of p14 is reduced by about 75%. This suggests that p18 consists of rod gamma, while p14 could be a mixture of cone gamma and rod gamma. The presence of serine residues in rod gamma might make the protein prone to phosphorylation and the charged protein might migrate in a different fashion, when compared to the unphosphorylated protein. So it appears that p18 could be a post-translational modification of rod gamma, while p14 could be a mixture of both isoforms.

Treatment with a specific serine/threonine phosphatase, PP2A treatment, cause p18 to disappear (Figure 5), further strengthening our argument that p18 is a post-translational modification of rod gamma.

We have shown that PDEγ inhibitory subunits are expressed in non-retinal tissues. The identification of the presence of transcript and the immunological detection of protein represent two independent methods for confirming the expression of these proteins in both testis and lungs. In humans, rod and cone PDEγ subunits are encoded by two separate genes. Data from the Gene Nomenclature Database suggest rod and cone subunits are encoded by two loci termed PDE6G and PDE6H. The rod PDEγ was cloned from human retinal photoceptors and was shown to encode an 87 aa containing protein[6]. It contains 4 exons. Exon 1 consists of promoter and transcriptional elements. Exons 2, 3 and 4 are spliced to produce the transcript (Figure 6). Analysing the nucleotide sequence of rod and cone gamma reveals that the sequences

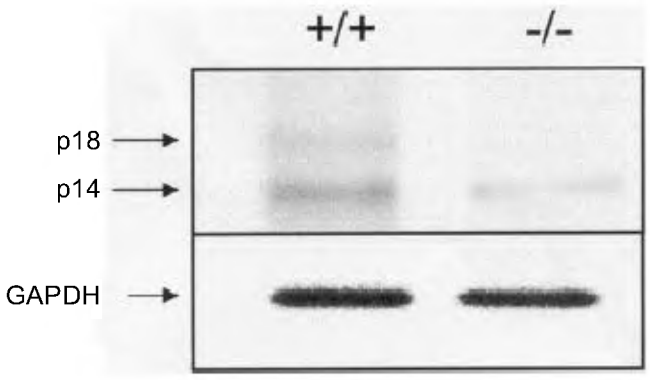

Figure 4. Immunoblotting with antibody raised against CPR give rise to two bands p14 and p18 in testicular tissues of wild type mice (PDE rod gamma +/+), while p18 is absent and p14 reduced to about 75% in homozygous knock-out mice. GAPDH levels indicate equal loading.

close to exon boundaries are identical and therefore both cone and rod gamma might have a similar exon organization in their encoder genes. Thus it seems that genes encoding cone and rod gamma have the same exon 3 but the exons 2 and 4 are unidentical. These findings are suggestive of evolutionary exon shuffling leading to the existence of two separate genes that encode rod and cone gamma respectively. The 41 bp deletion seen in short isoform of cone gamma represents an obvious case for alternate splicing. The 41 bp deletion observed in short isoform of cone gamma results in a frame change and the new in-frame stop codon might result in the early termination of translation resulting in a truncated protein, which has identical N-terminal and central polycationic region but different C-terminal region, when compared to that of the long isoform of cone gamma (Figure 6).

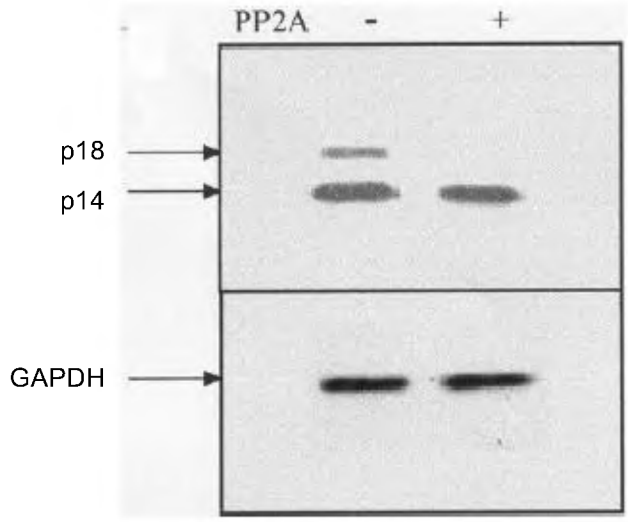

Figure 5. Treatment of anti-CPR immunoprecipitates from testis with PP2A abolishes p18, while p14 levels are unaffected. Stripping and probing with anti-GAPDH antibody indicates equal loading.

We have resolved the true nature of p14 and p18 first identified by Lochead *et al.* The treatment of immunoprecipitated PDEγ with serine/threonine-specific phosphatase PP2A eliminated p18, which indicates that p18 is a phosphorylated version of either rod or cone gamma (Figure 5). But immunoblotting of rod gamma –/– mice with antibodies raised against CPR of PDEγ shows that p18 is absent in rod gamma knock out mice (Figure 4). Therefore it is tempting to conclude that p18 is a phosphorylated form of rod gamma. 2-D gels coupled with

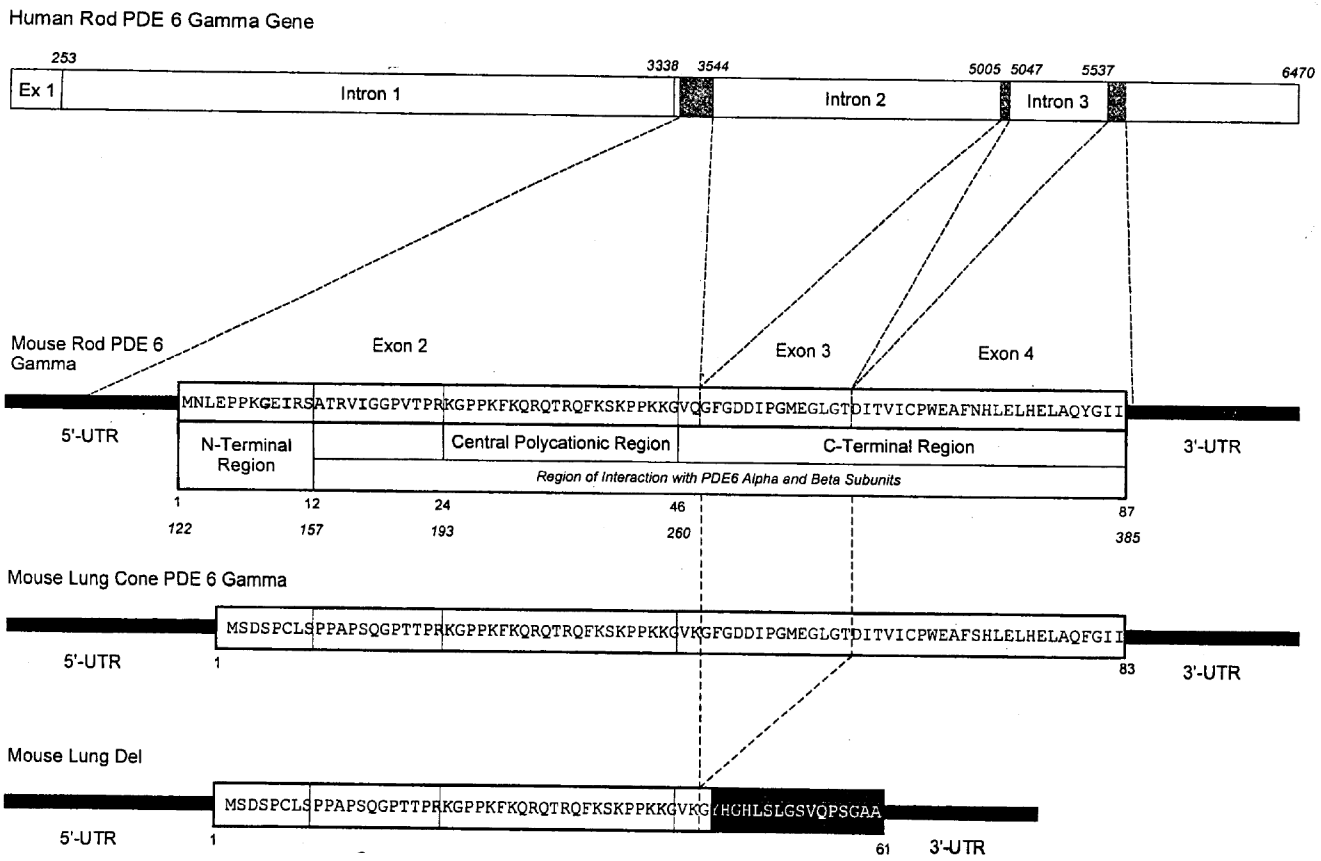

Figure 6. The genetic organization of human rod PDEγ. The protein sequence of rod gamma is compared with the predicted protein sequence of cone gamma. The central polycationic region of rod gamma is preserved in the long isoform, but not in the short isoform of cone gamma. This explains as to why antibodies raised against CPR recognizes rod gamma and long cone gamma. (Adapted from Tate, R. J. *et al.*, *Genomics*, 2002).

mass spectrometry analysis will be an ideal way to double-check this conclusion.

Lungs and testis are rich in PDE-5 and PDE-10 respectively. As explained earlier, these isoforms have striking similarity with PDE-6 and therefore we hypothesize PDEγ to interact with isoforms 5 and 10. Earlier reports suggest that recombinant rod PDEγ does not directly modulate the catalytic activity of PDE-5, but is instead specific for the PDE-5 activation by the PKA[4]. Also the mechanism by which PDEγ inhibits PKA-induced PDE-5 activity is likely to be different from the PDE-6 inhibition. It is shown that the C-terminal region of the PDEγ is responsible for the inhibition of PDE-6 catalytic activity, while CPR enhances the affinity of C-terminus to PDE-6 catalytic subunits. On the other hand, the CPR of PDEγ is responsible for the inhibition of PKA-induced PDE-5 activity and it maintains the inhibitory function even in the absence of C-terminus[7]. So further studies need to be done to elucidate the mechanism of this interaction.

A recent study has found that over-expression of recombinant rod and cone gamma in human embryonic kidney 293 cells enhances the activation of p42/p44 MAP kinase via EGF and thrombin[8]. The structural determinants in rod PDE that are involved in the interaction with dynamin II include Thr-62, which requires phosphorylation by G-protein coupled receptor kinase-2. Thr-62 is also conserved in the large cone isoform[5]. In addition, other structural determinants in rod PDE may also be important for binding to dynamin II. For instance, dynamin II interacts with SH3 domain containing proteins. In this regard, rod PDE contains an SH3 recognition consensus site at 20 PVTPRKGPP 28, which is identical with the corresponding region in the large cone isoform, except valine at amino acid position 21 that is replaced by threonine (Figure 5). The short cone PDE and rat ovary cone EST lack the Thr-62 phosphorylation site, but contain the SH3 binding site PTTPRKGPP. Therefore, the short versions may function either as weak activators of p42/p44 MAPK signaling or more likely as endogenous dominant-negative forms that compete with the larger PDE isoforms to block p42/p44 MAPK signaling.

Irrespective of the mechanism of interaction between PDEγ and isoforms other than PDE-6, the presence of these inhibitory subunits may have important pathophysiological role. Until date PDE-6 inhibitory subunits were only implicated in ophthalmological disorders such as progressive rod-cone degeneration[9]. Now that we have identified their presence in testis and lungs and as we are aware of their ability to inhibit the PDE-5 activity in airway smooth muscle it is tempting to speculate a role for these inhibitory subunits in the physiological activity of PDE-5 in lungs (bronchodilation[10]) and that of PDE-10 in testis (spermatogenesis[11]). New extra-retinal roles are being assigned to PDEγ. There is evidence to the role of PDEγ in caspase-3-mediated apoptotic responses[12]. Unpublished *in vitro* studies from the same researchers suggest that recombinant PDEγ can inhibit PKA-induced-PDE-5A-mediated metabolism of Atrial natriuretic peptide-induced cGMP in cultured kidney cells. As metabolism of cGMP by PDEs is important in regulating the signaling of natriuretic peptides and nitric oxide, understanding the role of PDEγ in modulating the respective PDE function may provide us new insights[13]. The visual transduction pathway is one of the most conserved signal transduction pathway in mammalians. The first G-protein-coupled receptor to be identified was rhodopsin and it later led to the identification of a huge family of GPCR. Similarly, other molecules such as arrestin and GTPase accelerating proteins, first identified in retinal phototransduction, were later identified in other tissues and important pathophysiological roles assigned[14]. Inhibitory subunit of PDE-6 follows the suit.

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