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Domain mapping of the retinal cyclic GMP phosphodiesterase γ-subunit

Function of the domains encoded by the three exons of the γ-subunit gene

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Retinal rod-outer-segment phosphodiesterase (PDE) is a heterotetramer consisting of two similar, but not identical, catalytic subunits (α and β) and two identical inhibitory subunits (γ_2). Previously, we have reported that the site of PDEα/β interaction with PDEγ is located within residues 54–87 [Cunnick, Hurt, Oppert, Sakamoto & Takemoto (1990) Biochem. J. **271**, 721–727]. The site for PDEγ interaction with transducin α (Tα) was found to encompass residues 24–45 of PDEγ [Morrison, Cunnick, Oppert & Takemoto (1989) J. Biol. Chem. **264**, 11671–11681]. In order to identify binding sites and other functional domains of PDEγ, the three peptides which are encoded by the three exons of the PDEγ gene were synthesized chemically. These exons encode for residues 1–49, 50–62 and 63–87 of bovine PDEγ [Piriev, Purishko, Khramtsov & Lipkin (1990) Dokl. Akad. Nauk. SSSR **315**, 229–230]. The peptide encompassing residues 63–87 was inhibitory in a PDE assay, whereas peptides 1–49 and 50–62 had no effect. However, both peptides 1–49 and 63–87 bound to PDEα/β in a solid-phase binding assay. Only peptide 1–49 bound to Tα·GTP[S] (GTP[S] is guanosine 5′-[γ-thio]triphosphate). These data confirm that the inhibitory region of PDEγ is encoded by exon 3 (residues 63–87), whereas a separate binding site for PDEα/β and for Tα·GTP[S] is encoded by exon 1 (residues 1–49). To study further the structure–function relationship of PDEγ, this entire protein and two mutants were chemically synthesized. One mutant (−CT) lacked residues 78–87, whereas another replaced tyrosine-84 with glycine (TYR-84). Whereas the synthetic PDEγ inhibited PDEα/β catalytic activity, the −CT and TVR-84 mutants did not. All three synthetic proteins bound to both PDEα/β and and Tα·GTP[S]. These data confirm the presence of an alternative binding site on PDEγ and demonstrate the importance of tyrosine-84 in PDEγ inhibitory activity.

INTRODUCTION

Visual transduction includes the events that occur from the absorption of a photon by a rhodopsin pigment molecule to the electrical response of the cell. The resulting changes in rhodopsin conformation induce the exchange of GDP for GTP on the α-subunit of the retinal guanine-nucleotide-binding (G-) protein, transducin (Tα) [1–7]. The Tα·GTP then binds to and removes the inhibitory subunit(s) of a cyclic GMP phosphodiesterase (PDEγ) from the two PDE catalytic subunits (PDEα/β) [8]. Upon lowering of cyclic GMP levels, a cell membrane cyclic GMP-binding channel protein is altered, and this results in membrane hyperpolarization [9,10].

The PDEγ subunit plays a pivotal role in visual transduction, and it must contain recognition regions for both of the PDEα/β catalytic subunits and for Tα. The sequence of this 87-amino-acid inhibitory protein is known for human, mouse and ox, and it is highly conserved [11–13]. There are only two conserved amino acid substitutions. Bovine PDEγ is *N*-terminally acetylated, but no other post-translational processing is known to occur [11]. The protein is multifunctional and binds to and inhibits Tα activity and PDEα/β activity [14–16].

The site of interaction with Tα has been identified through the use of synthetic peptides and lies within residues 24–45 [16,17]. Recently, recombinant constructs encoding PDEγ as a larger fusion protein have been expressed in *Escherichia coli* [18]. The fusion protein was cut with serum clotting factor Xa to yield a peptide which was identical with native PDEγ, except for *N*-terminal acetylation [18]. The inhibitory activity of the fusion protein was similar to that of native PDEγ, suggesting that the *N*-terminus is not responsible for inhibition. By contrast, cleavage of the *C*-terminus with carboxypeptidase Y [19] or a recombinant PDEγ which lacked residues 80–87 [18] resulted in proteins which did not inhibit PDEα/β. However, the recombinant protein did bind to PDEα/β [18].

In order to identify alternative binding regions and structural requirements for PDEγ, the peptides encoded by the three exons, the entire PDEγ, and two mutants were synthesized and tested for functional activity.

EXPERIMENTAL

Materials

Bovine eyes were obtained immediately after slaughter from Iowa Beef Packers (Emporia, KS, U.S.A.). Cyclic [8-^{3}H]GMP (15 Ci/mmol) was obtained from ICN Radiochemicals and purified further by anion-exchange chromatography. Carrier-free ^{125}I was purchased from Amersham International. Amino acids (t-butoxycarbonyl and resin forms) were obtained from Vega Biochemicals (United States Biochemical Corp., Cleveland, OH, U.S.A.) or Sigma. Aquacide III was purchased from Calbiochem, Vydac h.p.l.c. columns and TSK h.p.l.c. columns from P. J. Cobert Associates (St. Louis, MO, U.S.A.), nitrocellulose from Schleicher and Schuell, X-ray film from DuPont, and developing solutions from Kodak. Trypsin was from Sigma (bovine pancreas; EC 3.4.21.4; 12500 units/mg). Trypsin inhibitor (soybean type 1-S) was also from Sigma. All other buffers and reagents were from Sigma. Reagents used for peptide synthesis were h.p.l.c. grade from Fisher or Sigma. GTP[S] was from Boehringer-Mannheim.

Abbreviations used: KLH, keyhole-limpet haemocyanin; PDE, phosphodiesterase; PMSF, phenylmethanesulphonyl fluoride; ROS, rod outer segment(s); r.i.a., radioimmunoassay; GTP[S], guanosine 5′-[γ-thio]triphosphate; Tα, transducin-α; G-protein, guanine-nucleotide-binding protein.

Rod outer segment (ROS) purification

ROS were prepared by the method of Papermaster & Dreyer [20]. Bovine eyes were kept in the dark on ice during transport (about 3 h), and retinas were removed under dim red light and stored at −70 °C in the dark. All procedures were performed under dim red light unless noted otherwise. Approx. 50 thawed retinas were suspended in 50 ml of ROS # 1 [10 mM-Tris/HCl (pH 7.4)/65 mM-NaCl/2 mM-$MgCl_2$/1 mM-dithioerythritol (DTE)/0.5 mM-phenylmethanesulphonyl fluoride (PMSF) 30% sucrose (w/v)] and shaken vigorously for 1 min (resulting in ROS being sheared from the inner segments). After centrifugation at 4000 rev./min for 5 min (Sorvall SS-34 rotor; $r_{av.}$ 11 cm), the pellet was resuspended in 15 ml of ROS # 1 and re-centrifuged as above. Pooled supernatants were diluted 1:2 in ROS # 2 [10 mM-Tris/HCl (pH 7.4) 1 mM-$MgCl_2$/1 mM-DTE/0.5 mM-PMSF] and centrifuged at 7000 rev./min for 10 min (Sorvall SS-34 rotor). Pelleted ROS were resuspended in 12 ml of ROS # 3 [10 mM-Tris/HCl (pH 7.4)/1 mM-$MgCl_2$/1 mM-DTE/0.5 mM-PMSF/26.3% (w/w) sucrose], homogenized with a 26-gauge needle and layered (2 ml/tube) on to a discontinuous sucrose density gradient of 1 ml each of 1.11, 1.13, and 1.15 g/ml of sucrose in ROS # 2. After centrifugation at 24000 rev./min (55000 *g*) for 45 min (Beckman SW50.1 rotor), ROS discs were collected at the 1.11/1.13 g/ml sucrose interface. Purified ROS discs were then washed with 3 vol. of ROS buffer # 7 [10 mM-Tris/HCl (pH 7.4)/100 mM-NaCl / 5 mM-$MgCl_2$ / 0.1 mM-DTE / 1 mM-PMSF] and centrifuged at 15000 rev./min for 15 min (Sorvall SS-34 rotor). After washing two more times as above, the undepleted ROS membranes were resuspended in ROS # 7 and stored at −70 °C.

PDE purification

Undepleted ROS membranes (see above) was washed five times in ROS # 7 under dim red light and pelleted each time at 15000 rev./min for 15 min (Sorvall SS-34 rotor). Soluble PDE (non-activated) was eluted from the washed membranes by resuspending the pellet in 5 ml of ROS # 8 [10 mM-Tris/HCl (pH 7.4)/0.1 mM-DTE/0.2 mM-PMSF/10 μM-leupeptin/1 μM-pepstatin], homogenizing with a 26-gauge needle, incubating under bright light for 30 min on ice, and pelleting the membranes at 15000 rev./min for 15 min (Sorvall SS-34 rotor). The elution was repeated four times and the pooled supernatants containing soluble PDE were dialysed against h.p.l.c. buffer [20 mM-sodium phosphate (pH 6.8)/50 mM-Na_2SO_4/1 mM-DTE] and concentrated against Aquacide III. PDE was further purified by h.p.l.c. on a TSK G3000SW column (7.5 mm × 75 mm) using the same h.p.l.c. buffer. Fractions were monitored by their A_{280}, and PDE was identified by SDS/PAGE [21] and Western blotting (using peptide antisera to both catalytic and inhibitory subunits of ROS PDE).

PDEγ purification

PDEγ was purified from purified PDE (see above) by the method of Hurley & Stryer [22]. The pH of the PDE-containing supernatant was lowered by the addition of formic acid to a final concentration of 0.1 M. After heating at 70 °C for 5 min, the pH was re-adjusted to 6.5 (or until a precipitate formed) with dropwise addition of 4 M-NaOH. After centrifugation at 14500 rev./min for 15 min (Sorvall SS-34), the supernatant containing PDEγ was removed and the pellet resuspended in PDE buffer [50 mM-Tris/HCl (pH 7.2)/20 mM-$MgCl_2$] and the above procedure was repeated on the pellet. Pooled supernatants were then divided into portions and stored at −70 °C until utilized as the native PDEγ source.

Tα·GTP[S] purification

The above PDE-depleted membrane preparation was then used as a source of Tα by elution of these proteins in room light with 10 mM-Tris/HCl (pH 7.4)/1 mM-DTE/100 μM-GTP[S]/0.2 mM-PMSF/10 μM-leupeptin/1 μM-pepstatin. The crude Tα·GTP[S] was concentrated by an Amicon stirred ultrafiltration cell and further purified by h.p.l.c. on a TSK G2000 SW column by using a running buffer [20 mM-sodium phosphate (pH 6.8)/50 mM-Na_2SO_4/10 mM-$MgSO_4$/1 mM-DTE]. Purity of all proteins was assessed on Coomassie Blue-stained SDS/PAGE gels and on Western blots. Tα·GTP[S] contained 10–15% Tβγ after purification by h.p.l.c.

PDE activity assay

PDE activity was determined using the assay of Thompson & Appleman [23]. The final reaction mixture contained 50 mM-Tris, pH 7.4, 5 mM-$MgCl_2$, 40 μM-cyclic GMP, cyclic [^{3}H]GMP (40000 c.p.m./tube, sp. radioactivity 15 Ci/mmol) in a final volume of 400 μl. Reaction mixtures were incubated at 30 °C. Purified PDEγ and/or peptides were added as indicated.

In some cases, PDE was first activated with trypsin for 1 min on ice using 20 μg of purified PDE, 40 μg of trypsin in 400 μl final volume in buffer containing 50 mM-Tris/HCl, pH 7.4, and 20 mM-$MgCl_2$. The reaction was stopped by the addition of a twofold excess of trypsin inhibitor. The trypsin-treated PDE was then re-purified on a TSK G3000 SW h.p.l.c. column as described above to remove PDEγ fragments. After h.p.l.c. the PDE was assayed by radioimmunoassay to determine if all PDEγ had been removed using antisera to residues 1–49, 50–62 and 63–87 of bovine PDEγ. PDE which had all regions of PDEγ removed was used in binding assays.

Peptide synthesis and antisera production

Peptides corresponding to indicated PDEγ sequences were synthesized manually by the method of Merrifield [24] as modified by Gorman [25]. Cleavage of the peptide from the resin and protecting groups was accomplished with anhydrous HF [26].

The amino acid compositions of the peptides were determined by using reverse-phase h.p.l.c. with a Vydak C_{18} column and a 10–50% gradient of acetonitrile in 0.01 M-sodium phosphate, pH 7.0, and using *o*-phthalaldehyde as a detecting reagent [27]. Peptides (5 μl) were hydrolysed for 12 h under vacuum using 6 M-HCl at 110 °C, followed by freeze-drying to remove the HCl. Free amino acids were dissolved in 100 μl of water, and 10 μl aliquots were injected and quantified by comparison with known amino acid standards using a Shimadzu peak integrator.

Peptides corresponding to the three exon-encoded regions of PDEγ were purified using reverse-phase h.p.l.c. as discussed above. Synthetic PDEγ and mutants were purified on a TSK G2000 SW column as described for Tα·GTP[S] purification (see above). The fractions were monitored at 220 nm, by Western blots (Fig. 1), by Coomassie Blue staining (Fig. 2) and by PDE inhibition assays.

Peptide antisera were produced by cross-linking peptides to keyhole-limpet haemocyanin (KLH) by the procedure of Takemoto *et al.* [28]. Briefly, 0.2 ml of KLH (15 mg/ml) was added to 0.2 ml of peptide (5 mg/ml), followed by the addition of 0.2 ml of 15 mM-glutaraldehyde for cross-linking. The mixture was incubated with agitation at room temperature for 12 h. The reaction was quenched by the addition of 1 ml of $NaBH_4$ and then dialysed against 1 mM-ammonium bicarbonate, pH 7.0. Rabbits were injected initially with 200 μl of peptide–KLH with 200 μl of Freund's complete adjuvant, followed by biweekly injections of 200 μl of peptide–KLH with 200 μl of Freund's incomplete adjuvant. Sera were tested after 4 weeks by a ROS

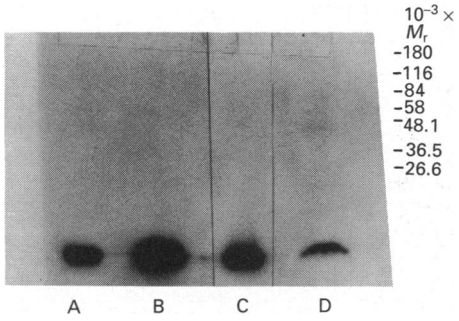

Fig. 1. Western blot of purified synthetic PDEγ species

Purified PDEγ proteins (~0.5 μg/lane) were separated on SDS/10%-PAGE and Western-blotted as described in the Experimental section. Reaction was with α 1–49 (see Table 1) antisera at 1:100. Proteins were revealed with ^{125}I-Protein A. Lane A, synthetic PDEγ; lane B, PDEγ *C*-terminal residues 78–87; lane C, PDEγ with tyrosine-84 replaced by glycine; lane D, bovine ROS preparation (10 μg of total protein).

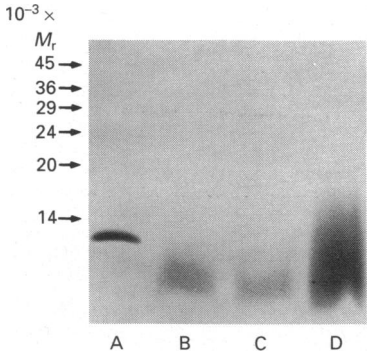

Fig. 2. Coomassie Blue stain of purified synthetic PDEγ species

Each lane was loaded with 0.5 μg of protein. The gel was a 10%-Tricine system [40]. Lane A, native PDEγ; lane B, synthetic PDEγ; lane C, −CT PDEγ; lane D, TYR-84 PDEγ. Note that synthetic PDEγ species are not *N*-terminally acetylated and migrate below native PDEγ, as does recombinant PDEγ [18].

Table 1. Antisera directory

Peptide antisera were produced by injecting rabbits subcutaneously with synthetic peptides linked to KLH (see the Experimental section). Bovine sequences for PDEα and PDEγ were as published [31,11]. To Tα sequence was also bovine [32].

	Peptide	
Antisera	Residues	Identity
α 1–49	1–49	PDE-γ
α 50–62	50–62	PDE-γ
α 63–87	63–87	PDE-γ
α NT	1–15	PDE-α
α TαC	1–15	T-α
α 740	740–755	PDE-α

protein Western blot and radioimmunoassays using the peptide the antisera was directed against native and retinal proteins. A directory of antisera is given in Table 1.

Protein determination

Protein concentrations were determined by the method of Bradford [29] or by scanning gel densitometry of Coomassie Blue-stained SDS/PAGE gels [21] using BSA standards. Gels were scanned on a Gilford multimedia densitometer using a Shimadzu integrator.

Radioimmunoassay binding assay

Solid-phase r.i.a. was a modification of the method of Suter [30]. Polystyrene tubes (12 mm × 75 mm) were incubated with 0.2% glutaraldehyde in 0.5 ml of buffer A (0.1 M-sodium phosphate, pH 5.0) and agitated at room temperature for 3 h. After washing three times with buffer A, initial peptides or proteins were added as described in the text in a volume to 200 μl of buffer B (0.1 M-sodium phosphate, pH 8.0). Tubes were incubated at 37 °C for 1 h, followed by washing twice with buffer C (0.15 M-NaCl/0.5% Tween 20) and once with buffer B. Purified PDEγ or peptides were then added to a final volume of 300 μl in buffer B and incubated while being agitated at room temperature. Tubes were then rinsed twice with buffer B and incubated with 0.2% glutaraldehyde in buffer A for 15 min under agitation at room temperature. This linked the protein or peptide covalently to the initial binding protein. After washing twice with buffer C and once with buffer D [0.1 M-sodium phosphate (pH 7.4)/0.15 M-NaCl/0.05% Tween 20], antisera as indicated was added at 1:100 in buffer D and tubes were incubated with agitation at room temperature overnight. Tubes were then washed three times with buffer E [10 mM-Tris (pH 8.0)/0.05% Tween 20], twice with water, and then 0.5 ml of buffer E containing ^{125}I-labelled Protein A (1×10^6 c.p.m./tube) was added to each tube and tubes were agitated for 1 h at room temperature. After washing twice with buffer E and twice with water, tubes were counted for radioactivity in a Beckman γ-radiation counter. The binding assays saturate at above 1 μg/assay tube. In all cases, controls are done to determine saturation levels and binding of individual peptides to tubes with glutaraldehyde.

Western blot

Proteins were separated by SDS/PAGE [21] and transferred to nitrocellulose using a Genie transfer apparatus (Idea Sci., Corvallis, OR, U.S.A.). After blocking in 2% BSA, blots were incubated with appropriate antisera, followed by washing and reaction with ^{125}I-Protein A. Exposure of the radioactive blots (usually overnight) to Cronex X-ray film and subsequent development revealed the proteins.

RESULTS

Exon peptides

The three peptides which represent the regions encoded by the exons of the PDEγ gene were synthesized. These are referred to as exon peptide 1 (residues 1–49), exon peptide 2 (residues 50–62) and exon peptide 3 (residues 63–87) [33]. Peptides were coupled to KLH and antisera were raised to each (Table 1). These antisera did not cross-react with unrelated peptides. Since exon peptide 1 was large, the epitope of the corresponding antiserum was determined by use of an r.i.a. using smaller peptides. The major epitope of α 1–49 is within residues 1–15 of PDEγ (results not shown). Individual antisera to the exon peptides did not cross-react with the other unrelated exon peptides.

These exon peptides and their specific antisera were used to measure the binding of each to PDEα/β or to Tα·GTP[S].

Exon peptides were first tested for their ability to inhibit the catalytic activity of a trypsin-activated h.p.l.c.-purified PDEα/β (Table 2). From these data it is apparent that only exon peptide 3 inhibited a trypsin-activated PDE. This confirms previous reports using a *C*-terminal deletion mutant of PDEγ [18] and using PDEγ peptides and proteolysis [19]. If a partially inhibited

Table 2. Effect of exon peptides on trypsin-activated PDE

The PDE assay was as described in the Experimental section using 0.06 μg of PDE/assay tube in 400 μl final volume. Trypsin activation was with 10 μg of trypsin for 1 min on ice, followed by 100 μg of trypsin inhibitor. This PDE was then purified by h.p.l.c. as described in the text. The PDE assay was for 5 min at 30 °C, using 40 μM-cyclic GMP and cyclic [^{3}H]GMP (30000 c.p.m./tube). Blank values (with no enzyme), have been subtracted from the samples. Max. is the maximum c.p.m. in the assay. Aliquots of 0.5 ml were counted by liquid-scintillation counting as described in [23].

Sample	Radioactivity (c.p.m.)	(% of control)
Max.	18330±618	
Blank	1823±332	
Control (not activated)	2725±252	
Trypsin-activated	8944±124	328
+Pγ-1* (μg)		
1	6172±857	226
5	7968±189	292
10	6375±440	233
25	6045±824	221
50	6306±767	231
75	7631±455	280
+Pγ-2 (μg)		
1	8613±722	316
5	7942±982	291
10	6763±731	248
25	6566±1692	240
50	5904±271	216
75	6359±480	233
+Pγ-3 (μg)		
1	6475±580	237
5	4343±679	160
10	4115±134	151
25	2734±996	100
50	2920±138	107
75	2745±692	100

* Exon peptides were added at the indicated levels (μg). Pγ-1, exon peptide 1, residues 1–49; Pγ-2, exon peptide 2, residues 50–62; Pγ-3, exon peptide 3, residues 63–87. Samples were run in triplicate and values are means±S.E.M.

Table 3. Effect of exon peptides on inhibited PDE

Experiments were performed as described in Table 2. Exon peptides were added to control PDE (not activated) and analysis was done as in Table 2. '% of control' is the percentage of the activity of the sample which was trypsin-activated and fully inhibited with Pγ. Results are means±S.E.M. for triplicate samples.

Sample	Radioactivity (c.p.m.)	(% of control)
Max.	29386±1067	
Blank	2037±578	
Control (not activated)	4206±677	
Trypsin-activated	14880±3549	
Trypsin-activated Pγ	3365±264	
+Pγ-1 (μg)		
5	2686±435	79
10	2774±60	82
15	3369±61	100
+Pγ-2 (μg)		
5	3248±10	96
10	2848±200	85
15	3244±200	96
+Pγ-3 (μg)		
5	2761±234	82
10	2258±35	67
15	1827±266	54

Table 4. Effect of exon peptides on activation of PDE by Tα·GTP[S]

Experiments were performed as described in Table 2. PDEα/β/γ_2 was used as 0.076 μg/assay tube and Tα·GTP[S] was used at 1.9 μg/assay tube. The Tα·GTP[S] was added 5 min before addition of Pγ-1, Pγ-2 or Pγ-3. Results are expressed as in Table 2.

Sample	Radioactivity (c.p.m.)	(% of control)
Max.	24116±483	
Blank	2469±519	
Control	4818±386	
Control+Tα·GTP[S]	18218±2040	378
+Pγ-1 (μg)		
5	15261±1435	316
10	13633±1412	282
25	15197±1011	315
50	13861±653	287
+Pγ-2 (μg)		
5	14611±337	303
10	14889±222	309
25	14197±222	294
50	12302±133	255
+Pγ-3 (μg)		
5	15226±316	316
10	14981±230	310
25	10895±309	224
50	8060±363	167

PDEα/β/γ_2 was used, none of the exon peptides could reverse this inhibition (Table 3). These results indicate that addition of the Tα·GTP[S]-binding peptide, exon peptide 1, is not sufficient to overcome inhibition by native PDEγ. By contrast, addition of exon peptide 3 further inhibited PDEα/β/γ_2, suggesting that not all of the PDE was α/β/γ_2. Since one of the PDEγ subunits has a low affinity for PDEα/β/γ [39], some of the PDEγ was probably lost in our preparation. Therefore, in this PDE preparation, further inhibition would occur by addition of excess exon peptide 3, as it mimicked the second PDEγ subunit.

The effects of the exon peptides on activation of PDEα/β/γ_2 by Tα·GTP[S] are illustrated in Table 4. Exon peptide 3 prevented activation by Tα·GTP[S], by acting at the inhibitory site. Exon peptides 1 and 2 had no effect, except at higher concentrations (slight effect of peptide 2 at 50 μg).

To determine which exon peptides bound to PDEα/β or to Tα·GTP[S], a solid-phase binding assay was utilized. Only exon peptide 1 bound to Tα·GTP[S] (Table 5). This binding was significantly above that of binding to the BSA used as a negative control. By contrast, both exon peptide #1 and 3 bound to PDEα/β (Table 6). These results demonstrate that PDEγ has an inhibitory region (residues 63–87) and a separate binding region (residues 1–49) for PDEα/β which are encoded by exons 1 and 3 of the PDEγ gene. By contrast, the binding site for Tα·GTP[S] is encoded by exon 1.

We have previously reported that the optimum binding site on PDEγ for Tα·GTP[S] is within residues 24–45 [17,19]. In order to determine the optimum binding site on PDEγ for PDEα/β, shorter segments of exon peptide 1 were synthesized and used in binding assays (Table 7). In this case, the peptide was linked to the tube, followed by binding of Tα·GTP[S] or PDEα/β. The

Table 5. Binding of exon peptides to Tα·GTP[S]

Binding assays were performed as described in the text using 1 μg/assay tube of Tα·GTP[S] and 1 μg of exon peptide 1 (1–49), 2 (50–62) or 3 (63–87)/assay tube. Reaction with indicated antisera, at 1:100, was with ^{125}I-Protein A added at 1×10^6 c.p.m./tube. BSA was used at 1 μg/tube. As a negative control, polystyrene tubes saturate above 1 μg/tube. Background is c.p.m. without Tα·GTP[S], exon peptide or antisera, and it has been subtracted from samples. C.p.m. are mean of four samples ± S.E.M. Antisera α 1–49, α 50–62, and α 63–87 are described in Table 1. Reactions of individual antisera with 1 μg of individual respective exon peptides/tube were, in c.p.m.: exon peptide 1 versus α 1–49, 10500 ± 1500 c.p.m.; exon peptide 2 versus α 50–62, 7300 ± 350 c.p.m.; exon peptide 3 versus α 63–87, 14600 ± 1750 c.p.m.

Antiserum	Sample	Radioactivity bound (c.p.m.)
α 1–49	Background	123 ± 8
	Tα·GTP[S]	1545 ± 79
	Tα·GTP[S] + 1–49	8669 ± 745
	BSA	487 ± 41
	BSA + 1–49	201 ± 89
α 50–62	Tα·GTP[S]	679 ± 78
	Tα·GTP[S] + 50–62	1300 ± 450
	BSA	834 ± 159
	BSA + 50–62	1670 ± 37
α 63–87	Tα·GTP[S]	391 ± 63
	Tα·GTP[S] + 63–87	1496 ± 110
	BSA	559 ± 187
	BSA + 63–87	1957 ± 185

Table 6. Binding of exon peptides to PDE

Experiments were performed as in Table 5. PDE was α/β, h.p.l.c.-purified and prepared as described in the text and used at 1 μg/tube. Results are means ± S.E.M. for four samples.

Antiserum	Sample	Radioactivity bound (c.p.m.)
α 1–49	Background	269 ± 30
	PDE	508 ± 72
	PDE + 1–49	10693 ± 1989
	BSA	709 ± 260
	BSA + 1–49	250 ± 128
α 50–62	PDE	708 ± 143
	PDE + 50–62	1581 ± 124
	BSA	1787 ± 247
	BSA + 50–62	1226 ± 461
α 63–87	PDE	819 ± 280
	PDE + 63–87	15792 ± 727
	BSA	3817 ± 167
	BSA + 63–87	2827 ± 780

binding region for Tα·GTP[S] included residues within peptides 16–30 and 31–45, however, the binding region for PDEα/β only included residues within peptide 31–45. This suggests that the Tα·GTP[S] and PDEα/β-binding sites are close and may overlap.

However, the inhibitory site on PDEγ is distinct (exon peptide 3, residues 80–87).

Synthetic PDEγ and mutants

In order to further define the structural requirements for PDEγ inhibition, the PDEγ and two mutants were synthesized entirely using Merrifield solid-phase methodology [24–27]. The results of purification and Western blots are illustrated in Figs. 1 and 2. By using the published bovine PDEγ sequence [11], synthetic PDEγ, a mutant lacking the last ten *C*-terminal amino acids, and a mutant in which tyrosine-84 was replaced by glycine, were synthesized. Amino acid analyses (results not shown) and radioimmunoassays (Table 8) were used to verify sequences.

These synthetic PDEγ proteins were tested for PDEα/β inhibition and for binding to PDEα/β and to Tα·GTP[S]. Only the synthetic PDEγ inhibited PDEα/β catalytic activity (Table 9). No inhibition was observed for the *C*-terminal deletion protein or for the tyrosine-84 mutant. The synthetic PDEγ did not work as well as native PDEγ. Whereas native PDEγ inhibited PDEα/β fully at 0.13 μg/assay, for a similar inhibition more

Table 7. Binding of Tα·GTP[S] or PDEα/β to regions of exon peptide 1

Binding assays were with 1 μg of the indicated peptide corresponding to bovine PDEγ residues 1–15, 16–30, 31–45 or 46–60/tube. Either 1 μg of PDEα/β or 1 μg of Tα·GTP[S] was then added, incubated as described in the text and glutaraldehyde-fixed. Binding was measured using indicated antisera (see Table 1) at 1:100. Samples are means ± S.E.M. for four samples.

Antiserum	Sample	Radioactivity bound (c.p.m.)
αTαC	Background	113 ± 12
	Pγ1–15	125 ± 41
	Pγ1–15 + Tα·GTP[S]	437 ± 123
	Pγ16–30	349 ± 155
	Pγ16–30 + Tα·GTP[S]	1505 ± 66
	Pγ31–45	529 ± 101
	Pγ31–45 + Tα·GTP[S]	1479 ± 208
	Pγ46–60	168 ± 14
	Pγ46–60 + Tα·GTP[S]	266 ± 89
α NT	Pγ1–15	624 ± 56
	Pγ1–15 + PDE	573 ± 121
	Pγ16–30	288 ± 75
	Pγ16–30 + PDE	420 ± 92
	Pγ31–45	417 ± 78
	Pγ31–45 + PDE	1843 ± 7
	Pγ46–60	135 ± 7
	Pγ46–60 + PDE	138 ± 29

Table 8. Radioimmunoassays of synthetic PDEγ species

Samples were added at 1 μg/tube and allowed to react with indicated antisera at 1:100. Radioactivity-bound values are means ± S.E.M. for four samples. Abbreviations: P-γ-S, total bovine synthetic PDE-γ; P-γ-CT, total bovine PDE-γ minus last 10 *C*-terminal amino acids; P-γ-84, total bovine PDE-γ with tyrosine-84 replaced by glycine.

Antiserum	Sample	Radioactivity bound (c.p.m.)
α 1–49	Background	262 ± 15
	P-γ-S	1472 ± 56
	P-γ-CT	1999 ± 90
	P-γ-84	2196 ± 82
α 50–62	Background	196 ± 28
	P-γ-S	1479 ± 44
	P-γ-CT	926 ± 161
	P-γ-84	1509 ± 135
α 63–87	Background	153 ± 29
	P-γ-S	1103 ± 139
	P-γ-CT	245 ± 57
	P-γ-84	2225 ± 780

Table 9. Effect of synthetic PDEγ species on trypsin-activated PDE

PDE assays were as described in Table 2. PDE was at 0.025 μg/assay and native PDEγ was at 0.13 μg. P-γ-S, P-γ-CT and P-γ-84 are as described in Table 8. Results are means ± S.E.M. for three samples.

Sample		Radioactivity (c.p.m.)	(% of control)
Max.		28739 ± 1442	
Blank		5883 ± 812	
Control (not activated)		7324 ± 983	
Trypsin-activated		22260 ± 908	303
+ PDE-γ	(0.13 μg)	10653 ± 1193	145
+ P-γ-S	(0.13 μg)	19961 ± 227	272
	(0.32 μg)	20173 ± 529	275
	(1.3 μg)	15006 ± 587	204
	(2.6 μg)	13338 ± 1279	182
+ P-γ-CT	(0.72 μg)	20035 ± 908	273
	(2.9 μg)	19303 ± 210	263
	(5.8 μg)	25175 ± 2124	343
	(173 μg)	21794 ± 464	297
+ P-γ-84	(0.121 μg)	20674 ± 1138	282
	(1.21 μg)	20144 ± 231	275
	(3.02 μg)	19412 ± 571	265
	(194.0 μg)	22060 ± 74	301

Table 10. Binding of synthetic PDEγ species to PDE or to Tα·GTP[S]

Binding assays utilized 1 μg of either Tα·GTP[S] or h.p.l.c.-purified and trypsin-activated PDEα/β per tube. the synthetic PDEγ species (see Table 9) were then added at 1 μg/tube and incubated and fixed as described in the text. Reaction was with α 1–49 at 1:100. Results are means ± S.E.M. for three samples.

Sample		Radioactivity bound (c.p.m.)
Tα·GTP[S]		381 ± 69
Tα·GTP[S]	+ P-γ-S	890 ± 166
	+ P-γ-CT	728 ± 193
	+ P-γ-84	1825 ± 62
PDEα/β		1258 ± 305
PDEα/β	+ P-γ-S	4402 ± 426
	+ P-γ-CT	3622 ± 177
	+ P-γ-84	4263 ± 214

than 2.6 μg/assay of synthetic PDEγ was required (Table 9). This may be because less of the synthetic protein attains the correct conformation.

Binding assays demonstrated that all three of the synthetic PDEγ proteins bound to both PDEα/β and Tα·GTP[S] (Table 10). This is presumably because the binding region (residues 1–49) had not been changed in the mutants. This suggests that a PDEγ which does not inhibit PDEα/β can still bind to it. These results further demonstrate that tyrosine-84 of PDEγ is critical for inhibitory activity. Binding of the tyrosine-84 mutant to Tα·GTP[S] appears to be greater than to the synthetic PDEγ or mutant. This is despite their equal binding to PDEα/β (Table 10). This may indicate that interactions occur at the sites within exon peptide 1 and 3.

DISCUSSION

Although many of the initial events in phototransduction are well understood, the mechanism by which Tα·GTP activates the ROS PDE is not [1–7]. The PDE is composed of related catalytic α- and β-subunits [31,34] and a γ-subunit which acts as an inhibitor [14,35–37]. The PDEγ subunit inhibits PDEα/β catalytic activity by decreasing the $V_{max.}$ without altering the K_m [36]. The holoenzyme consists of an $\alpha/\beta/\gamma_2$ complex [14,35–37], and full activation of the PDE to α/β requires the removal or displacement of both subunits [19,38]. This is accomplished via a direct interaction of Tα·GTP[S] with the PDEγ subunit [19]. Both high- and low-affinity binding sites have been demonstrated for PDEγ on the PDEα/β [39].

The discovery of two PDEγ subunits, each with different binding affinities and with possible allosteric control [39], suggests that a highly specific mechanism controls PDE activity. In the bovine retina, removal of the low-affinity PDEγ, with low levels of Tα·GTP[S], results in only a 5% PDE$\alpha/\beta/\gamma$ activation [39]. Full activation requires 2–4 μM-Tα·GTP[S], and this level of activator does not result in the actual removal of the remaining PDEγ from the membrane [19].

PDEγ is 87 amino acids long [11], is highly conserved [11–13] and is multifunctional. It binds to both PDEα/β and to Tα·GTP, and alters the activity of both [17,22]. PDEγ inhibits PDEα/β catalytic activity and Tα·GTPase activity [17,22]. The site of interaction with Tα·GTP has been identified through the use of synthetic peptides and resides in a basic domain, including residues 24–45 [17,19].

Recombinant constructs encoding the PDEγ as a fusion protein have been expressed in *E. coli* [18,41,42]. The inhibitory activity of the recombinant PDEγ was identical with that of native PDEγ. The fusion protein, which had 31 extra amino acids at the *N*-terminus, was also active, suggesting that the last ten amino acids are not required for inhibitory activity. In contrast, cleavage of the *C*-terminus with carboxypeptidase Y [19] or a recombinant PDEγ which lacked residues 80–87 [18] did not inhibit PDEα/β catalytic activity. This demonstrates that the *C*-terminus is required for inhibitory activity. Since the *C*-terminal-deletion mutant still bound to PDEα/β [18], an alternative binding site probably exists.

Recently, the bovine gene for PDEγ has been sequenced and found to contain three exons which code for residues 1–49, 50–62 and 63–87 [33]. We have used solid-phase peptide synthesis to produce these three exon peptides chemically. PDE activity assays demonstrated that the inhibitory region of PDEγ is within exon peptide 3 (residues 63–87), as previously suggested [18,19]. Binding assays demonstrated that the binding region for both Tα·GTP[S] and for PDEα/β was in exon peptide 1 (residues 1–49). These results indicate that PDEγ has both an inhibitory and a separate binding region for PDEα/β which are coded for by different exons. The fact that the binding regions on PDEγ for both Tα·GTP[S] and for PDEα/β are close, and overlap, suggests that some competition may occur between PDEα/β and Tα·GTP[S] for PDEγ.

PDEγ was chemically synthesized and found to be almost as active as native PDEγ. A *C*-terminal deletion protein and a protein in which tyrosine-84 was replaced by glycine were also chemically synthesized. This is the first reported total chemical synthesis of active PDEγ. The *C*-terminal deletion protein did not inhibit PDEα/β catalytic activity, but still bound to PDEα/β and to Tα·GTP[S]. This demonstrates that an inactive PDEγ can still bind to PDEα/β and to Tα·GTP[S].

Of particular interest is the tyrosine-84 mutant. This protein no longer inhibited PDEα/β, but still bound to PDEα/β and to Tα·GTP[S]. This demonstrates that this residue is critical for inhibitory function.

In conclusion, we have domain-mapped PDEγ and have identified binding sites for Tα·GTP[S] (residues 24–45) and for PDEα/β (residues 30–45). In addition, these data demonstrate

that the tyrosine residue (tyrosine-84) is required for inhibitory activity.

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