Identification of a binding site on retinal transducin α for the phosphodiesterase inhibitory γ subunit

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Transducin alpha ($T\alpha$) activates retinal rod cyclic GMP phosphodiesterase (PDE) by interacting with and removing the inhibitory $PDE\gamma$ subunit. A $T\alpha$ – $PDE\gamma$ complex can be isolated *in vitro*, and our previous work [Morrison, Rider and Takemoto (1987) FEBS Lett. **222**, 266–270; Morrison, Cunnick, Oppert and Takemoto (1989) J. Biol. Chem. **264**, 11671–11681] has identified a region of $PDE\gamma$, residues 24–45, that binds to $T\alpha$. The C-terminal region of $PDE\gamma$ is the site that interacts with $PDE\alpha/\beta$ and inhibits catalytic function. The site on $T\alpha$ that binds to the $PDE\gamma$ 24–45 region has not been identified. Synthetic peptides (15-mers) which span the bovine $T\alpha$ sequence were tested for binding to purified recombinant $PDE\gamma$ using a solid-phase assay. The peptides were also tested for ability to activate a PDE complex.

We have identified a region, residues 250–275 of $T\alpha$, which shows a high affinity for $PDE\gamma$ and for the $PDE\gamma$ (24–45) binding peptide. The peptide did not bind to the C-terminal residues 50–87 of $PDE\gamma$. Likewise, a region of $T\alpha$, 1–25 did not exhibit high-affinity binding to $PDE\gamma$ or to the 24–45 $PDE\gamma$ peptide. Specific binding of the 250–275 peptide to $PDE\gamma$ was confirmed by its ability to compete with $T\alpha$ binding to $PDE\gamma$, although a higher concentration was required ($10\times$). The $T\alpha$ -(250–275) peptide activated a fully inhibited $PDE\alpha\beta\gamma_2$ complex in a dose-dependent manner. These results suggest that a region on $T\alpha$ that recognizes the $PDE\gamma$ -binding site is found within residues 250–275 of $T\alpha$.

INTRODUCTION

In photoreceptor cells, visual transduction is realized through the photoexcitation of rhodopsin and the resultant exchange of GDP for GTP on the retinal G-protein, transducin (T) [1–7]. The $T\alpha$ –GTP then binds to the inhibitory subunit of the phosphodiesterase (PDE), forming a soluble $T\alpha$ – $PDE\gamma$ complex [8–10] or a $T\alpha$ – $PDE\alpha\beta\gamma_2$ complex [11,12]. The nature of this complex depends on the occupancy of the non-catalytic cyclic GMP (cGMP)-binding sites found on the PDE α and β catalytic subunits [13]. If these sites are occupied, then the $T\alpha$ forms a complex with the $PDE\alpha\beta\gamma_2$; if the sites are empty, the $T\alpha$ removes the $PDE\gamma$ from the $PDE\alpha\beta$ [13]. On activation of the PDE, the resulting lowering of cGMP levels results in changes in the cGMP-gated channel protein. This results in membrane hyperpolarization [14,15].

Physiologically, this means that at high cGMP levels, in the dark, the non-catalytic sites would be occupied and $PDE\gamma$ would be bound tighter to the $PDE\alpha\beta$, resulting in low basal activity. When cGMP is low, in the light, the $PDE\gamma$ would bind less tightly. The GTPase activity that terminates the PDE activity is slower when these sites are occupied and the $T\alpha$ is in a complex with the $PDE\gamma$ [16,17].

The $PDE\gamma$ subunit plays a pivotal role in the visual transduction cascade as it must have binding sites for both PDE α and β and for the $T\alpha$. The sites have been identified, and mutants have been tested to verify these functional residues [18–22]. The basic region of $PDE\gamma$ encompassing residues 24–45 contains a binding site for $T\alpha$ and for the $PDE\alpha\beta$, whereas the inhibitory site and a lower-affinity site for $T\alpha$ can be found within the C-terminus. Recently, a site for phosphorylation by rod outer segment (ROS) protein kinase C has been identified on Thr-35

[23]. The phosphorylated $PDE\gamma$ has increased ability to inhibit the PDE activity.

It has been postulated that, like the PDE catalytic subunits [24,25], the $T\alpha$ subunits may have two sites for interaction with $PDE\gamma$ [26]. One region which interacts with the C-terminus of the $PDE\gamma$ is included in residues 293–314 of $T\alpha$ [26]. The region of interaction of $T\alpha$ that recognizes the basic $PDE\gamma$ region of residues 24–45 has not been identified. By use of synthetic peptides and a direct binding assay, this region has been identified as including residues 250–275 of $T\alpha$.

EXPERIMENTAL

Materials

Bovine eyes were obtained immediately after slaughter from Iowa Beef Packers (Emporia, KS, U.S.A.). [$8\text{-}^3\text{H}$]cGMP (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 Sigma. Aquacide III was purchased from Calbiochem, h.p.l.c. columns from P. J. Cobert (St. Louis, MO, U.S.A.), nitrocellulose from Schleicher and Schuell, X-ray film from DuPont, and developing solutions from Kodak. All other buffers and reagents were from Sigma. Reagents used for peptide synthesis were h.p.l.c. grade from Fisher or Sigma, or Sequenal grade from Pierce.

PDE purification

ROS were prepared by the method of Papermaster and Dreyer [27]. Purified ROS discs were washed with buffer containing

Abbreviations used: ROS, rod outer segments; PDE, cyclic GMP phosphodiesterase from rod outer segments; $T\alpha\beta\gamma$, subunits of the rod outer segment guanine nucleotide-binding protein, transducin; $PDE\alpha\beta\gamma$, subunits of the phosphodiesterase; GTP[S], guanosine 5'-[γ -thio]triphosphate; cGMP, cyclic GMP; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol.

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10 mM Tris/HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 1.0 mM dithioerythritol and 0.2 mM phenylmethanesulphonyl fluoride (PMSF). Soluble PDE (non-activated) was eluted from the washed membranes with 10 mM Tris/HCl, pH 7.4, containing 1.0 mM dithiothreitol (DTT), 0.2 mM PMSF and 2 μ M leupeptin. Soluble PDE was dialysed against h.p.l.c. buffer (20 mM sodium phosphate, pH 6.8, 100 mM Na₂SO₄, 1 mM DTT) and concentrated against Aquacide III or by Amicon ultrafiltration. PDE was further purified by h.p.l.c. on a TSK G3000SW column using the same h.p.l.c. buffer. Fractions were monitored by absorbance at 280 nm and PDE was identified by SDS/PAGE [28] and Western blotting [10] using peptide antisera to both catalytic and inhibitory subunits of ROS PDE.

PDE γ purification

PDE γ was expressed in *Escherichia coli* and purified as described [20]. The PDE γ was utilized as a fusion protein with 38 residues of λ cII and the clotting-proteinase (factor Xa) cleavage site and the PDE γ sequence (87 residues) [20]. PDE γ was purified by reversed-phase chromatography on a h.p.l.c. Vydak C-4 column using a 10–90% acetonitrile gradient in 0.1% trifluoroacetic acid.

T α -GTP[S] purification

PDE-depleted ROS membranes were then used as a source of T α by elution of these proteins with 10 mM Tris/HCl, pH 7.4, buffer containing 1 mM DTT, 100 μ M GTP[S], 0.2 mM PMSF and 2 μ M leupeptin. The crude T α -GTP[S] was concentrated against Aquacide III or by Amicon ultrafiltration and further purified by h.p.l.c. on a TSK G2000 SW column using a running buffer containing 20 mM sodium phosphate (pH 6.8), 50 mM Na₂SO₄, 10 mM MgSO₄ and 1 mM dithioerythritol. T β γ was purified under the same conditions. Purity of all proteins was assessed on Coomassie-Blue-stained SDS/polyacrylamide gels and on Western blots.

Isolation of the T α 33 kDa fragment

Purified T α -GTP[S] was incubated for 45 min on ice with 16 μ g of T α /unit of insoluble trypsin (Tos-Phe-CH₂Cl 'TPCK'-treated trypsin/acrylamide; Sigma) in 50 mM Tris/HCl, pH 7.2, 5 mM MgCl₂. Insoluble Tos-Phe-CH₂Cl-treated trypsin was removed by centrifugation. After proteolysis, the amount of 33 kDa compared with whole T α was assessed by gel scanning and scanning of autoradiograms using antiserum to the 15 C-terminal residues of T α (α T α C), and to residues no. 52–66 of T α [α T α -(52–66)]. The preparation was usually less than 5% T α . To remove T α , the 33 kDa piece was separated by h.p.l.c. gel filtration, resulting in pure 33 kDa (no detectable T α). The 33 kDa piece still contained GTP[S] as assessed by the use of [³⁵S]GTP[S]. The fragment still activates PDE [29], but does not bind to rhodopsin or to T β γ [30–33].

PDE activity assay

PDE activity was determined using the assay of Thompson and Appleman [34]. The final reaction mixture was 50 mM Tris/HCl, pH 7.4, 5 mM MgCl₂, 40 μ M cGMP and [³H]cGMP (40 000 c.p.m./tube, specific radioactivity 15 Ci/mmol) in a final volume of 400 μ l. Reaction mixtures were incubated at 30 °C. Purified PDE γ and/or peptides were added as indicated.

In some cases, PDE was first activated with trypsin for 1 min on ice using 20 μ g of purified PDE and 40 μ g of trypsin in 400 μ l

final volume in buffer containing 50 mM Tris/HCl (pH 7.4) and 20 mM MgCl₂. The reaction was stopped by the addition of 2 $\times$ excess trypsin inhibitor. The trypsin-treated PDE was then repurified on an h.p.l.c. TSK G3000 SW column as described above to remove PDE γ fragments. After h.p.l.c. the PDE was assayed by radioimmunoassay to determine if all PDE γ had been removed using antisera to residues 1–49 [α P γ -(1–49)], 50–62 [α P γ -(50–62)] and 63–87 (α P γ C) of bovine PDE γ [35]. PDE which had all regions of PDE γ removed was used in binding assays.

Peptide synthesis and antiserum production

Peptides corresponding to indicated bovine amino acid sequences were synthesized by the method of Merrifield [36] as modified by Gorman [37]. Cleavage of the peptide from the resin and protecting groups was accomplished with anhydrous HF [38]. Peptides were purified and quantified using reversed-phase h.p.l.c. [10]. Sequences were verified using amino acid analyses [39] and mass spectrometry.

Peptide antisera were produced by cross-linking peptides to keyhole limpet haemocyanin by the procedure of Takemoto et al. [40].

Protein determination

Protein concentrations were determined by the method of Bradford [41] or by scanning gel densitometry of Coomassie Blue-stained SDS/polyacrylamide gels [28] using BSA standards. Gels were scanned on a Gilford multimedia densitometer using a Shimadzu integrator.

Radioimmunoassay/binding assay

Solid-phase radioimmunoassay was a modification of the method of Suter [42]. Polystyrene tubes (12 mm $\times$ 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 the tubes had been washed three times with buffer A, initial peptides or proteins were added as described in the text in a volume to 200 μ l of buffer B (0.1 M sodium phosphate, pH 8.0). Tubes were incubated at 37 °C for 1 h, followed by two washes with buffer C (0.15 M NaCl, 0.5% Tween 20) and one with buffer B. Purified proteins or peptides were then added to a final volume of 300 μ l 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 with agitation at room temperature. This linked the protein or peptide covalently to the initial binding protein. After two washes with buffer C and one with buffer D (0.1 M sodium phosphate, pH 7.4, 0.15 M NaCl, 0.05% Tween 20), antisera as indicated were 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/HCl, pH 8.0, 0.05% Tween 20), and twice with water, and then 0.5 ml of buffer D containing ¹²⁵I-labelled protein A (10⁶ c.p.m./tube) was added to each tube and the tubes were agitated for 1 h at room temperature. After two washes with buffer E and two with water, tubes were counted in a Beckman γ counter. The significance of binding of a secondary polypeptide to a tube-bound polypeptide was determined by comparison with the binding of that secondary polypeptide to a blocked blank tube ($P = 0.05$).

Western blot

Proteins were separated by SDS/PAGE [28] and transferred to nitrocellulose using a Genie transfer apparatus (Idea Science,

Corvallis, OR, U.S.A.). After being blocked in 2% BSA, blots were incubated with appropriate antisera, followed by washing and reaction with ^{125}I -Protein A.

RESULTS

We have previously reported that the three exons of PDE γ that encode residues 1–49, 50–62 and 63–87 [43] represent distinct functional regions of the protein [10]. The region with the most

Table 1 Binding of retinal visual transduction proteins to PDE γ

Binding assays were performed as described in the text using 2 pM/assay tube of T α -GTP[S], T α 33 kDa or PDE γ . Antisera were used at 1:100 dilution and were quantified using ^{125}I -Protein A at 10^6 c.p.m./tube. Polystyrene tubes were saturated at above 1 μg /tube. Background (302 ± 21 c.p.m.) is without protein or peptides and is subtracted from the samples. Values are means $\pm$ S.E.M. of three samples. Antiserum $\alpha\text{P}\gamma(1-49)$ is used. Reaction of antiserum with 2 pM of PDE γ , directly bound to the tube, was 19160 ± 950 c.p.m. The reaction of $\alpha\text{P}\gamma(1-49)$ to PDE γ added to a Tween-20-blocked tube without bound protein was 840 ± 156 c.p.m. PDE γ was added to bound protein.

Sample	Radioactivity bound (c.p.m.)
T α -GTP[S] alone	681 ± 172
T α -GTP[S] + PDE γ	10907 ± 782
T α 33 kDa alone	329 ± 42
T α 33 kDa + PDE γ	5235 ± 804

Table 2 Binding of PDE γ to T α peptides

Binding assays were as described in Table 1 using T α -GTP[S] at 2 pM/tube and peptides at 30 pM/tube. PDE γ was added as the second (binding) protein at 0.22 pM/tube. Antiserum [$\alpha\text{P}\gamma(1-49)$] was used at 1:100 dilution with ^{125}I -Protein A at 10^6 c.p.m./tube. PDE γ before blocking = 4500 ± 190 c.p.m.; PDE γ after blocking = 430 ± 23 c.p.m. Numbers refer to amino acids residues of the bovine T α sequence [44]. Background (92 ± 30 c.p.m.) is without protein or peptide and is subtracted from the samples. Values are means $\pm$ S.E.M. of three samples.

Peptide	Radioactivity bound (c.p.m.)
T α -(1–15)	405 ± 19
T α -(16–30)	231 ± 78
T α -(31–45)	118 ± 9
T α -(46–60)	131 ± 22
T α -(61–75)	612 ± 186
T α -(76–90)	730 ± 8
T α -(91–105)	614 ± 158
T α -(96–110)	896 ± 186
T α -(106–120)	123 ± 19
T α -(121–135)	718 ± 30
T α -(136–150)	315 ± 32
T α -(151–165)	348 ± 13
T α -(166–180)	754 ± 3
T α -(181–195)	778 ± 201
T α -(196–210)	102 ± 24
T α -(211–225)	774 ± 268
T α -(226–240)	610 ± 62
T α -(241–255)	1046 ± 317
T α -(256–270)	2625 ± 659
T α -(271–285)	767 ± 16
T α -(286–300)	608 ± 208
T α -(301–315)	715 ± 146
T α -(316–330)	838 ± 33
T α -(331–345)	654 ± 184
T α -(336–350)	731 ± 51

Table 3 Binding of PDE γ to T α peptides

Binding assays were as described in Table 1. T α -GTP[S], PDE γ and peptides were added as indicated in parentheses. Order of samples indicates order of addition to tube. Antiserum [$\alpha\text{P}\gamma(1-49)$] was used at 1:100 dilution with ^{125}I -Protein A at 10^6 c.p.m./tube. 'Before' indicates sample added before blocking, i.e. bound to the tube. 'After' indicates sample added after blocking with Tween 20. Values are means $\pm$ S.E.M. of three samples.

Sample	Radioactivity bound (c.p.m.)
PDE γ – before (0.02 nM)	30674 ± 3026
PDE γ – after (0.02 nM)	1665 ± 685
T α -GTP[S] alone – before (5.4 nM)	778 ± 146
T α -GTP[S] + PDE γ (as above)	25305 ± 117
T α -(1–45) alone – before (5.4 nM)	263 ± 35
T α -(1–45) + PDE γ	1896 ± 847
T α -(293–314) alone – before (9.6 nM)	263 ± 35
T α -(293–314) + PDE γ	4195 ± 319
T α -(250–275) alone – before (1.2 nM)	2886 ± 276
T α -(250–275) + PDE γ	13447 ± 332

energy of binding for both T α and PDE $\alpha\beta$ is found within a basic region, residues 24–45, and the inhibitory region is within residues 80–87 [10]. It appears that two sites are also utilized by the α and β subunits of the PDE for interaction with PDE γ [24,25]. It was of interest to see if T α , which also interacts directly with PDE γ , has two sites of interaction. One site has been identified within residues 293–314 of T α [26], but this region interacts with the C-terminus of PDE γ . In order to identify the region of T α that interacts with the basic region of PDE γ (residues 24–45), whole T α -GTP[S] was tested in a direct binding assay as well as the 33 kDa fragment of T α . This T α fragment has been shown to activate PDE but does not bind to rhodopsin or to T $\beta\gamma$ [29–33]. Binding to PDE γ was observed using this direct binding assay with both T α and the 33 kDa fragment (Table 1). However, binding was reduced for 33 kDa T α indicating that either cleavage disrupts the native structure or that the N- and C-termini which are not included within the 33 kDa piece are also required for optimum binding of PDE γ .

We have previously used a rapid screening technique to identify potential regions of proteins that bind to PDE γ [24]. This is feasible for PDE γ because the binding site within the basic region is linear and has little secondary structure (J. Cunnick, C. Twamley, I. Udovichenko, K. Gonzalez and D. J. Takemoto, unpublished work). This approach was also used to tentatively identify regions of T α that bind to whole PDE γ (Table 2).

As the initial screen used peptides that were 15 amino acids in length, larger peptides were used to rescreen the regions that were thought to contain a binding site for PDE γ . These included T α -(1–45), T α -(293–314) and a larger region of a tentative binding site, T α -(250–275). Very high binding was observed for the T α -(250–275) peptide whereas lower binding was observed for the previously identified region (Table 3). In contrast, the N-terminal peptide T α -(1–45) did not exhibit binding to PDE γ (compare with 'PDE γ – after', i.e. PDE γ added after the tube with no bound protein or peptide was blocked).

A major binding region of PDE γ is within residues 24–45 of PDE γ [10] and we have used the peptide that corresponds to residues 1–49 of PDE γ to determine if this is the region that is recognized by T α -(250–275). This peptide, PDE γ -(1–49), was found to bind to whole T α -GTP[S] as well as whole PDE γ bound to T α -GTP[S] (Table 4). PDE γ -(1–49) bound both to T α -(250–275) and to whole T α (Table 5). In contrast, PDE γ -(1–49) did not bind to T α -(293–314) or T α -(1–45) peptides [compare

Table 4 Binding of T α to PDE γ or PDE γ -(1-49)

Binding assays were described in Table 1. T α -GTP[S] was added first at 25 nM/tube followed by addition of PDE γ or PDE γ -(1-49) as indicated. Antiserum [α P γ -(1-49)] was used at 1:100 dilution with 125 I-Protein-A at 10⁶ c.p.m./tube. PDE γ (114 nM/tube) before block = 27246 $\pm$ 285 c.p.m.; PDE γ (114 nM/tube) after block = 1675 $\pm$ 357 c.p.m.; PDE γ -(1-49) (204 nM/tube) before block = 43021 $\pm$ 1019 c.p.m.; PDE γ -(1-49) (204 nM/tube) after block = 1407 $\pm$ 185 c.p.m. Background (333 $\pm$ 14 c.p.m.) is without protein or peptide and is subtracted from the samples. Values are means $\pm$ S.E.M. of three samples.

Sample	Radioactivity bound (c.p.m.)
T α -GTP[S] alone	908 $\pm$ 155
T α -GTP[S] + PDE γ (11 nM)	7821 $\pm$ 115
(23 nM)	10976 $\pm$ 211
(90.0 nM)	15448 $\pm$ 850
T α -GTP[S] + PDE γ -(1-49) (10 nM)	7491 $\pm$ 197
(20 nM)	11931 $\pm$ 104
(102 nM)	19319 $\pm$ 383

Table 5 Binding of T α peptides to PDE γ peptides

Binding assays were as described in Table 1. T α -GTP[S] was added at 0.02 nM/tube, T α -(1-45) at 5.4 nM, T α -(293-314) at 9.6 nM, T α -(250-275) at 5.9 nM, PDE γ -(1-49) at 5.9 nM. Antiserum [α P γ -(1-49)] was added at 1:100 dilution with 125 I-Protein-A at 10⁶ c.p.m./tube. The backgrounds for each antiserum in tubes without protein or peptide have been subtracted from the samples. Background for α P γ -(1-49) was 291 $\pm$ 96 c.p.m. and background for α P γ C was 410 $\pm$ 158 c.p.m. Values are means $\pm$ S.E.M. of three samples.

Antiserum	Sample	Radioactivity bound (c.p.m.)
α P γ -(1-49)	PDE γ -(1-49) - before	35253 $\pm$ 728
	PDE γ -(1-49) - after	1745 $\pm$ 39
	PDE γ -GTP[S] alone - before	897 $\pm$ 54
	T α -GTP[S] + PDE γ -(1-49)	13195 $\pm$ 2453
	T α -(1-45) alone - before	430 $\pm$ 228
	T α -(1-45) + PDE γ -(1-49)	1492 $\pm$ 123
	T α -(293-314) alone - before	261 $\pm$ 27
	T α -(293-314) + PDE γ -(1-49)	1505 $\pm$ 239
	T α -(250-275) alone - before	1817 $\pm$ 261
	T α -(250-275) + PDE γ -(1-49)	8282 $\pm$ 1801
α P γ C	PDE γ -(50-87) alone - before	2096 $\pm$ 47
	PDE γ -(50-87) alone - after	565 $\pm$ 131
	T α -(250-275) alone - before	830 $\pm$ 59
	T α -(250-275) + PDE γ -(50-87)	680 $\pm$ 53

with 'PDE γ -(1-49) - after' in Table 5, i.e. binding of PDE γ -(1-49) to a blocked tube without bound protein or peptide]. T α -(250-275) did not bind to the C-terminal peptide, PDE γ -(50-87). This indicates that the T α -(250-275) binds only to the binding region [PDE γ -(1-49)] of PDE γ .

If T α -(250-275) binds specifically to PDE γ , then it should prevent or decrease the binding of T α -GTP[S] to PDE γ . Competition binding assays were conducted using the ability of the added peptide to prevent the binding of PDE γ to the T α -GTP[S] which was bound to the assay tube (Table 6). No competition was observed using the peptides T α -(1-45) or T α -(293-314). However, T α -(250-275) completely prevented the binding of T α -GTP[S] to PDE γ at 0.12 nM. This was about 10-fold excess of peptide over T α and PDE γ .

If the T α -(250-275) peptide competes for T α -GTP[S] binding to PDE γ , then it may activate the inhibited PDE through interaction with the inhibitory PDE γ subunits. In order to test

Table 6 Competition by T α peptides for binding of PDE γ by T α -GTP[S]

Binding assays were as described in Table 1 using α PDE γ -(1-49) at 1:100 dilution and 10⁶ c.p.m./tube for 125 I-Protein A. 'Before' and 'After' refer to additions before or after blocking with Tween 20. PDE γ and T α -GTP[S] were used at 0.02 nM/tube. Peptides were added at the same time as the PDE γ to compete, concentrations are indicated in parentheses. Background (302 $\pm$ 21 c.p.m.) is without protein or peptide and is subtracted from the samples. Values are means $\pm$ S.E.M. of three samples.

Sample	Radioactivity bound (c.p.m.)
PDE γ alone - before	19160 $\pm$ 949
PDE γ alone - after	842 $\pm$ 156
T α -GTP[S] alone - before	681 $\pm$ 172
T α -GTP[S] + PDE γ	10907 $\pm$ 782
T α -(1-45) - before	255 $\pm$ 112
T α -(293-314) - before	246 $\pm$ 53
T α -(250-275) - before	1470 $\pm$ 618
T α -GTP[S] + PDE γ + T α -(1-45) (1 nM)	9270 $\pm$ 1612
(2 nM)	9669
(3.2 nM)	10945
(4.3 nM)	9086
(5.4 nM)	9176
(27.0 nM)	11874
T α -GTP[S] + PDE γ + T α -(293-314) (0.96 nM)	10818 $\pm$ 1402
(2.8 nM)	11773 $\pm$ 23
(5.7 nM)	8496 $\pm$ 526
(9.6 nM)	7896 $\pm$ 109
(19.2 nM)	7843 $\pm$ 688
(28.8 nM)	9281 $\pm$ 789
T α -GTP[S] + PDE γ + T α -(250-275) (0.12 nM)	421 $\pm$ 160
(0.24 nM)	552 $\pm$ 104
(0.60 nM)	1005 $\pm$ 242

this, a PDE activity assay was carried out using PDE that was fully activated, then re-inhibited by the addition of excess PDE γ . PDE inhibited by adding PDE γ could be activated by the addition of T α -GTP[S] (Table 7). The addition of an equal concentration of T β γ or an excess of T α -(1-45) (27000 pM) did not activate the PDE. However, the peptide T α -(250-275) activated the PDE at 1200 pM. This was about 50 times as much as was required for activation by T α -GTP[S] (27 pM).

DISCUSSION

The structure of transducin, the GTP-binding protein of the retinal ROS, has not been determined in detail. It consists of α , β and γ subunits of molecular mass 39 kDa, 37 kDa and 6 kDa respectively in a T α β γ complex [1-7].

During visual transduction, the photoexcited rhodopsin undergoes a conformational change which results in exchange of GDP for GTP on the T α subunit. T α is then released from T β γ which remains membrane associated. The T α subunit activates PDE α β γ by directly interacting with and removing the constraints of the inhibitory PDE γ subunits [8-12].

A structural model has been proposed for T α which consists of domains for interactions with Mg²⁺-GDP and -GTP, T β γ , rhodopsin and PDE [45]. This model was developed using previously known sequence data and information obtained from partial proteolysis of T α [30-32]. T α can be functionally divided into a 1-2 kDa region on the N-terminus, which is needed for binding to T β γ [30-33], a 21 kDa region (approximately residues 19-204), which interacts with the PDE [33], a 12 kDa region, which binds guanine nucleotides, and a C-terminus, which binds to rhodopsin [32,46]. This model has, thus far, not been confirmed by tertiary structural analyses.

Table 7 Effect of $T\alpha$ peptides on phosphodiesterase activity

PDE was measured as described in the Experimental section using 0.78 pM pure PDE and 22 pM PDE γ . PDE was either not activated (control) or was trypsin activated to remove PDE γ , reconstituted with PDE γ (22 pM), and repurified on h.p.l.c. Trypsin activation was as described for 1 min on ice. Assay was for 10 min at 30 °C with 20 μ M cGMP. Total protein concentration is in parentheses. Results are means $\pm$ S.E.M. of three samples. The blank (i.e. background; 4358 $\pm$ 640 c.p.m.) without protein or peptide was subtracted from the samples. The maximum radioactivity possible was 18550 $\pm$ 588 c.p.m.

Sample	PDE activity (c.p.m.)
Control PDE	9304 $\pm$ 926
Trypsin-activated PDE	13224 $\pm$ 579
Trypsin-activated PDE + PDE γ	8905 $\pm$ 571
+ $T\alpha$ -GTP[S] (27 pM)	11437 $\pm$ 262
+ $T\beta\gamma$ (20 pM)	8253 $\pm$ 821
+ $T\alpha$ -(1–45) (542 pM)	8626 $\pm$ 1103
(27000 pM)	8315 $\pm$ 698
+ $T\alpha$ -(250–275) (60 pM)	8778 $\pm$ 969
(120 pM)	9580 $\pm$ 552
(1200 pM)	14772 $\pm$ 1045

The identity of the site for interaction of $T\alpha$ with PDE γ is of interest because this interaction constitutes the initial activation of PDE by $T\alpha$ -GTP. We have previously functionally mapped the PDE γ -inhibitory protein using partial proteolysis, mutants and synthetic peptides [10,18–21]. The 87-amino-acid-length protein has a high-affinity binding region in residues 24–45 and an inhibitory region at the C-terminus. This protein interacts with the PDE α/β at both sites [10]. Likewise, both PDE α and β have two interaction domains for PDE γ [24,25].

The location of the $T\alpha$ -binding sites in the 21 kDa piece is an approximation. In fact, recent structural studies have identified one site of $T\alpha$ interaction with the C-terminus of PDE γ on $T\alpha$ residues 293–314 [26]. We now report that another site is found within $T\alpha$ residues 250–275. This region binds to PDE γ at the 'basic region' binding site, but not at the C-terminus. The peptide, $T\alpha$ -(250–275), also competes for $T\alpha$ -GTP[S] binding to PDE γ and activates PDE. These results imply that, like PDE α/β , $T\alpha$ also has two interaction domains for PDE γ . The binding mechanism is not known, but the major energy of interaction of PDE γ with $T\alpha$ is within the 24–45 region of PDE γ [22]. As previously suggested [26], perhaps the initial interaction of $T\alpha$ -(293–314) at the C-terminus of PDE γ lowers the binding affinity at the second site [$T\alpha$ -(250–275) for PDE γ -(24–45)]. There are also modifications on both PDE α/β [13] and PDE γ [23] which control the binding affinity of $T\alpha$ for PDE γ . The occupancy of the non-catalytic cGMP-binding sites on PDE α/β determines whether $T\alpha$ removes PDE γ or remains in a $T\alpha$ -PDE $\alpha/\beta\gamma_2$ complex [13]. In addition, we have recently reported [23] that PDE γ is phosphorylated at Thr-35 by retinal protein kinase C. The phosphorylated PDE γ has increased ability to interact with and inhibit PDE α/β [23]. When phosphorylated, the PDE γ also shows decreased binding to $T\alpha$ -(250–275) (J. Cunnick, C. Twamley, I. Udovichenko, K. Gonzalez and D. J. Takemoto, unpublished work).

Thus $T\alpha$ appears to have two sites for interaction with PDE γ , and the interactions of these domains are under multiple control mechanisms.

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