Binding of the γ -subunit of retinal rod-outer-segment phosphodiesterase with both transducin and the catalytic subunits of phosphodiesterase

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The γ -subunit of retinal rod-outer-segment phosphodiesterase (PDE- γ) is a multifunctional protein which interacts directly with both of the catalytic subunits of PDE (PDE α/β) and the α -subunit of the retinal G (guanine-nucleotide-binding)-protein transducin α (T α). We have previously reported that the PDE γ binds to T α at residue nos. 24–45 [Morrison, Rider & Takemoto (1987) FEBS Lett. **222**, 266–270]. *In vitro* this results in inhibition of T α GTP/GDP exchange [Morrison, Cunnick, Oppert & Takemoto (1989) J. Biol. Chem. **264**, 11671–11681]. We now report that the inhibitory region of PDE γ for PDE α/β occurs at PDE γ residues 54–87. This binding results in inhibition of either trypsin-solubilized or membrane-bound PDE α/β . PDE γ which has been treated with carboxypeptidase Y, removing the C-terminus, does not inhibit PDE α/β , but does inhibit T α GTP/GDP exchange. Inhibition by PDE γ can be removed by T α -guanosine 5'-[γ -thio]triphosphate (GTP[S]) addition to membranes. This results in a displacement of PDE γ , but not in removal of this subunit from the membrane [Whalen, Bitensky & Takemoto (1990) Biochem. J. **265**, 655–658]. These results suggest that low levels of T α -GTP[S] can result in displacement of PDE γ from the membrane *in vitro* as a GTP[S]-T α -PDE γ complex. Further activation by high levels of T α -GTP[S] occurs by displacement of PDE γ from its inhibitory site on PDE α/β , but not in removal from the membrane.

INTRODUCTION

Retinal rod outer segments respond to light by decreasing membrane current [1]. This ion channel is gated by cyclic GMP which is, in turn, controlled by synthesis via guanylate cyclase and degradation via a specific cyclic GMP phosphodiesterase (PDE) [2,3].

The cyclic GMP PDE is made up of three subunits which exist as an $\alpha\beta\gamma_2$ complex [4,5]. M_r values for the α , β and γ subunits determined by SDS/PAGE are 88 000, 84 000, and 11 000 respectively [4]. The PDE γ subunit (PDE γ) inhibits PDE catalytic activity through a decrease in V_{max} , leaving the K_m unaltered [6,7]. The full activation of PDE α/β requires the removal by transducin α (T α)-GTP of both PDE γ subunits [8]. Each PDE γ binds with a different affinity and, in the bovine retina, removal of the low-affinity PDE γ results in a 5–17% activation of the PDE α/β [8,9]. The second high-affinity PDE γ is removed at high concentrations of T α -GTP and results in full activation of PDE α/β [8,9]. Evidence suggests that T α -GTP may activate the PDE α/β by directly interacting with PDE γ to facilitate its removal from PDE α/β [7,8]. Removal of the low-affinity PDE γ requires lower amounts of T α -GTP and results in the release of a soluble guanosine 5'-[γ -thio]triphosphate (GTP[S])-T α -PDE γ complex *in vitro* [8,9]. This may or may not occur *in vivo*. At high levels of T α -GTP[S] full activation of PDE α/β occurs, but this does not result in the release of a GTP[S]-T α -PDE γ complex [8,9]. Presumably T α , PDE α/β and PDE γ remain bound to the membrane and activation occurs via some displacement mechanism. Proof of this hypothesis has been hindered by a lack of a means of identifying individual proteins on the membrane and by a lack of methodology for obtaining a fully-activated PDE α/β enzyme which remained membrane-bound. Partial trypsin digestion of PDE $\alpha\beta\gamma_2$ leads to the degradation of PDE γ and subse-

quent activation of the PDE [6]. However, this treatment results in the irreversible removal of PDE α/β from the membrane [10] and in an altered K_m as well as the characteristic increase in V_{max} [6,7]. We have used carboxypeptidase Y to obtain a membrane-bound PDE α/β which is inhibited by PDE γ and reactivated by T α -GTP[S]. This has been used to study the inactivation process and to identify the binding sites of PDE γ for T α and for PDE α/β .

EXPERIMENTAL

Materials

Fresh bovine eyes were obtained from a local slaughterhouse (Iowa Beef Packers, Emporia, KS, U.S.A.). Buffers, GTP, GDP and other reagents were from Sigma. GTP[S] was from Boehringer-Mannheim. [γ - 32 P]GTP (50 Ci/mmol), and [35 S]GTP[S] (1200 Ci/mmol), were from NEN/du Pont. Cyclic [8- 3 H]GMP (19 Ci/mmol) was from ICN Radiochemicals. Carrier-free 125 I was from Amersham International. The t-butoxycarbonyl-amino acids and their resins were from Vega Biochemicals, United States Biochemicals or from Sigma. Aquacide III was from Calbiochem. All other reagents used for peptide synthesis were h.p.l.c.-grade from Fisher or Sigma, or Sequenal grade from Pierce. All materials used for SDS/PAGE were from Bio-Rad or Sigma.

Preparation of rod outer segments

Rod outer segments were prepared by the method of Papermaster & Dreyer [11]. Fresh bovine eyes were obtained from a local slaughterhouse within 60 min of slaughter. The eyes were transported in the dark and on ice. Retinas were removed under dim red light and stored in the dark without buffer at -70°C .

Abbreviations used: PDE, 3',5'-cyclic nucleotide phosphodiesterase; BME, β -mercaptoethanol; PMSF, phenylmethanesulphonyl fluoride; DTE, dithioerythritol; G-protein, guanine-nucleotide-binding protein; GTP[S], guanosine 5'-[γ -thio]triphosphate; T α and T β , transducins α and β .

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Typically a preparation consisted of 50–100 dark-adapted retinas thawed quickly in the dark. All purification steps were done in dim red light and on ice unless otherwise indicated. The rod outer segments were isolated from the total retina as previously described [12].

Purified rod outer segments were removed from the gradient at the 1.11–1.13 g/ml interface and washed with ROS-#7 [10 mM-Tris (pH 7.4)/100 mM-NaCl/5 mM-MgCl₂/0.1 mM-dithioerythritol (DTE)/1 mM-PMSF]. The rod outer segments were pelleted at 15000 rev./min for 15 min in an SS-34 rotor. This procedure was repeated twice more. The washed pellet was resuspended in GTPase buffer [10 mM-Mops (pH 7.4)/2 mM-MgCl₂/1 mM- β -mercaptoethanol (BME)/0.1 mM-phenylmethanesulphonyl fluoride (PMSF)] for subsequent assays. The rod-outer-segment preparation was stored at -70°C and is referred to herein as an undepleted rod-outer-segment preparation.

Preparation of depleted membranes and retinal proteins

Depleted rod-outer-segment membranes were prepared from the undepleted membrane preparation described above. Briefly, the undepleted membrane preparations were washed five times in 10 mM-Tris/HCl (pH 7.4)/0.1 M-NaCl/5 mM-MgCl₂ under dim red light at 4°C . The membranes were pelleted each time at 15000 rev./min for 15 min in a Sorvall SS-34 rotor. Soluble PDE $\alpha/\beta/\gamma$ was eluted from the membrane preparation under room light with 10 mM-Tris/HCl (pH 7.4)/0.5 mM-MgCl₂/0.1 mM-DTE/0.2 mM-PMSF/10 μM -leupeptin/1 μM -pepstatin. This procedure was repeated three times. If preparations were to be subsequently treated with proteinases, the proteinase inhibitors were omitted. The soluble PDE $\alpha/\beta/\gamma$ was further purified by concentrating the eluate with Aquacide III. The concentrate was separated on a Vydak TSK G3000SW h.p.l.c. column using a running buffer containing 20 mM-sodium phosphate, pH 6.8, 50 mM-Na₂SO₄ and 1 mM-DTE. Fractions were monitored at 280 nm, and purity was assessed on Coomassie Blue-stained SDS/PAGE gels and on Western blots using antisera to PDE α and to PDE- γ .

The above PDE-depleted membrane preparation was then used as a source of T α 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. This procedure was repeated three times. The crude transducin preparation was concentrated by Amicon ultrafiltration and further purified by HPLC on a Vydak TSK G2000 SW column using a running buffer with 20 mM-sodium phosphate (pH 6.8)/50 mM-Na₂SO₄/10 mM-MgSO₄/1 mM-DTE. Purity was assessed on Coomassie Blue-stained SDS/PAGE gels and on Western blots using antisera to T α and T β .

The remaining depleted rod-outer-segment membrane preparation was further depleted by washing four times in 10 mM-Tris/HCl (pH 7.4)/1 mM-DTE/1 mM-EDTA. The final wash was with 10 mM-Tris/HCl (pH 7.4)/0.1 mM-DTE. The EDTA-washed membranes were suspended in the later buffer and utilized as a source of rhodopsin and of membrane-bound PDE $\alpha/\beta/\gamma$ for membrane reconstitution experiments. In some cases, this membrane preparation was washed once with 5 M-urea. Soluble PDE γ was prepared as previously described [10] using a crude PDE α - β - γ complex.

GTPase assay

The GTPase assay was performed as previously described [13]. Typically, 4 μg of undepleted rod outer segments were used per 100 μl of GTPase buffer. Reactions were initiated by the addition of GTP to a final concentration of 20 μM -GTP (20 μM -GTP+0.1 μCi of [γ -³²P]GTP per assay tube). The reaction mixture was incubated under room light at 37°C for 1–5 min,

then terminated by the addition of 1 ml of a solution containing 150 mM-HClO₄/0.1 mM-KH₂PO₄ and 1 ml of a solution containing 10 mM-ammonium molybdate and 20 mM-triethylamine/HCl. The resulting precipitate was collected on a Whatman GF/A glass-microfibre filter using a suction-filtration apparatus. Each filter was washed with 50 ml of a solution containing 10 mM-triethylamine/HCl/200 mM-HClO₄/2.5 mM-ammonium molybdate. The quantity of [³²P]P_i released from the γ -phosphate of GTP was determined by liquid-scintillation counting. In some experiments peptides or PDE γ were preincubated with the undepleted rod-outer-segment membranes for 5 min at 37°C before the addition of substrate.

PDE assay

PDE activity was determined by the method of Thompson & Appleman [14]. The final concentrations in the reaction mixture were 50 mM-Tris/HCl (pH 7.4), 5 mM-MgCl₂, 40 μM -cyclic GMP and cyclic [³H]GMP (150 000 c.p.m./tube; specific radioactivity 19 Ci/mmol) in a final volume of 400 μl . Reactions took place at 30°C . Purified PDE γ or other proteins were added as indicated.

Membrane-binding assays and proteinase treatments

Various amounts of EDTA-depleted rod-outer-segment membranes were incubated at 30°C with either buffer A [50 mM-Tris/HCl (pH 7.4)/5 mM-MgCl₂] or purified T α , T β γ or PDE γ in buffer A for 5 min. Membranes were pelleted in a Microfuge at 16000 rev./min for 1 min and washed twice in buffer A. Membrane pellets were assayed for PDE activity and for protein content on a Western blot [15].

In some cases, either PDE γ or the EDTA-depleted membranes were preincubated with carboxypeptidase Y (from yeast; 0.1 unit/ μl of assay mixture), insoluble trypsin (from bovine pancreas; 0.1 unit/ μl of assay mixture) or cathepsin C (from bovine spleen; 0.1 unit/ μl of assay mixture). All enzymes were from Sigma. Incubation was for 5 min at 30°C unless otherwise indicated. Membrane pellets were washed with 10 mM-Tris/HCl (pH 8.4)/1 mM-DTE/1 mM-PMSF twice, then once in buffer without PMSF.

If PDE γ , T α -GTP[S] or T $\alpha/\beta/\gamma$ was added to these membranes, incubation with exogenous purified proteins (twice the maximum capacity of the preparation) was for 5 min at 30°C . Membranes were then washed twice with 10 mM-Tris/HCl, pH 7.5, and used in subsequent PDE assays. In all cases, membranes were assayed for total amount of protein which could be recovered in the pellet. This varied with each membrane preparation.

Peptide synthesis and purification and antisera production

Peptides corresponding to 15-amino-acid-long segments of bovine PDE γ or other retinal proteins were synthesized manually by the method of Hodges & Merrifield [16] as modified by Gormann [17]. Cleavage of the peptide from the resin and protecting groups was accomplished with anhydrous HF.

Peptides used in inhibition assays were purified by cellulose thin-layer electrophoresis in a buffer containing acetic acid/formic acid/water (3:1:16, by vol.). Peptides were spotted on to the thin-layer cellulose plates and dried. Buffer was then sprayed on to the thin-layer cellulose plates and electrophoresed in the same buffer at 1000 V until the tracking dye, Pyronin-Y, had migrated 9 cm. After electrophoresis, the plate was dried and the peptide was revealed with ninhydrin. Corresponding unsprayed lanes were scraped off and the peptide was eluted with aq. 0.5% NH₃. The peptides were then freeze-dried and dissolved in distilled water. The pH of the peptides was adjusted to 7.4 with 2 M-Tris, pH 11 [12].

Peptides were quantified or further purified by reverse-phase

h.p.l.c. using a Vydak C-18 column and a 10–50% gradient of acetonitrile in 0.01 M-sodium phosphate, pH 7.0, using *o*-phthalaldehyde as a detecting reagent [18]. Peptides (5 μ l aliquots) were hydrolysed for 12 h *in vacuo* using 6 M-HCl at 110 °C. The HCl was removed by freeze-drying and the amino acids dissolved in 100 μ l of water. Portions (10 μ l) were analysed for amino acid content and were quantified by comparison with known amino acid standards using a Shimadzu peak integrator.

Peptide antisera were produced and characterized as previously described [15]. Antisera utilized were designated as follows and all correspond to bovine sequences: PDE γ -6, against residues 73–87 of PDE γ ; SAg-2 against residues 225–239 of S-antigen; T α C, against residues 326–350 of T α ; T β C, against residues 326–340 of T β ; PDE- α NT, against residues 1–15 of PDE α .

Quantification

Protein concentrations were determined by the method of Bradford [19] or Lowry *et al.* [20] using BSA as a standard. SDS/PAGE was as described [21]. Individual proteins were quantified by scanning of either Coomassie Blue-stained gels using BSA as a standard or of autoradiograms, using individual purified proteins as standards. Gels or autoradiograms were scanned on a Gilford Multimedia densitometer using a Shimadzu integrator. Unless otherwise indicated, all gels were 7.5% acrylamide.

RESULTS

We have previously described the synthesis of peptides of bovine PDE γ [22]. These peptides were designated PDE γ -1–

PDE γ -6. Each of these peptides was tested for inhibition of GTPase activity of T α [12,22]. We have previously reported that PDE γ -3, corresponding to bovine PDE γ residues 31–45, constituted the optimum PDE γ -binding region for T α [12,22]. In Table 1 are listed the results of a similar experiment utilizing these peptides to identify the PDE γ inhibitory site for inhibition of PDE. When a trypsin-activated PDE is utilized as a source of 100%-activated enzyme, this enzyme can be reinhibited by intact PDE γ [6]. We have used this same approach with synthetic peptides which correspond to regions of PDE γ . The peptide which best inhibits PDE activity is PDE γ -6. More specifically, when smaller peptides are used, it appears that most of the inhibitory activity resides in residues 80–87. This agrees with previous reported results [23,24]. To confirm further these results, intact PDE γ was treated with a C-terminal-specific proteinase, carboxypeptidase Y. This proteinase will cleave amino acid residues sequentially from the C-terminus of PDE γ . The treated PDE γ no longer inhibits PDE activity. However, this treated PDE γ still inhibits T α GTPase activity, presumably because that binding site (residues 31–45) has not been cleaved off in this short-term proteolysis (Table 2). These results indicate that PDE γ is a multifunctional protein with different binding sites for PDE α/β (residues 80–87) and for T α (residues 31–45). It further suggests that this protein may have a binding site for T α and PDE α/β which may compete (within residues 24–45).

It was formerly thought that the PDE holoenzyme consisted of an $\alpha/\beta/\gamma$ structure. However, recent data suggest that it actually exists as an $\alpha-\gamma-\beta-\gamma$ complex [5]. But γ subunit binds with a different affinity [8,9]. The presence of less than 1 μ M-T α -GTP[S] is reported to activate the PDE by only 5–17% and to remove one PDE γ subunit. Full activation required the presence of at least 4 μ M-T α -GTP[S] [8,9]. We have attempted to study the T α -GTP[S]-induced activation of membrane-bound PDE using highly purified proteins and a depleted rod-outer-segment membrane preparation as a source of intrinsic PDE. The proteins present after depletion of a rod-outer-segment membrane preparation with 1 mM-EDTA or 5 M-urea are shown in Fig. 1. It is apparent that, even after extensive washing of rod-outer-segment membranes, there remains considerable PDE α and PDE γ as assessed by reaction with antisera in a Western blot. In these membrane preparations rhodopsin typically constituted 95% of the membrane protein. The preparation shown in Fig. 1 contained 0.74 μ g of rhodopsin/ μ l, 0.0027 μ g of PDE γ / μ l and 0.0024 μ g of PDE α/β / μ l. Little or no T $\alpha\beta\gamma$ was detected. There was also considerable S-antigen remaining bound to these membranes. This preparation was used as a source of PDE to study the activation of PDE by T α -GTP[S]. Whereas removal of the first,

Table 1. Effect of PDE γ peptides on PDE activity

PDE activity was measured as described [14], using 40 μ M-cyclic GMP at 30 °C for 5 min in a final volume of 400 μ l. The trypsin-activated and inhibited PDEs were prepared as described in the Experimental section. Quantities, in 400 μ l (final volume) of assay mixture, were 0.32 μ g of trypsin-activated PDE or 0.4 μ g of inhibited PDE. PDE γ was prepared as described in the Experimental section and 0.35 μ g was added per assay. The PDE γ (treated) sample was identical with PDE γ , but was preincubated for 5 min at 30 °C with 0.1 μ g of carboxypeptidase-Y. 0.5 mM-PMSF was added to stop proteolysis. This PDE γ preparation was used at 0.35 μ g/assay. Peptides (Pyl etc.) were added to the assay before the substrate. Under 'Peptide sequence', residue numbers refer to the amino acid sequence of bovine PDE γ [27] as numbered from the N-terminus. Under 'Activity', – means not done; '% of control' refers to PDE activity compared with trypsin-activated PDE (taken as 100%). Peptides were added at 200, 400 or 800 μ g/assay. Results are means for four samples.

Sample	Peptide sequence	Peptide added (μ g)...	Activity (% of control)		
			200	400	800
Trypsin-activated	–		100	100	100
Inhibited	–		63	55	33
+ P γ 1	1–14		–	–	110
+ P γ 2	16–30		–	–	90
+ P γ 3	31–45		–	101	107
+ P γ 4	46–60		–	98	112
+ P γ 5	61–74		–	–	70
+ P γ 6	73–87		–	–	29
+ P γ -54	54–60		97	70	101
+ P γ -61	61–72		98	89	132
+ P γ -73	73–79		112	87	72
+ P γ -80	80–87		101	67	53
PDE- γ	–		27	28	–
PDE- γ (treated)	–		107	102	–

Table 2. Effect of PDE γ on T α GTPase activity

The GTPase activity of T α was measured as described in the Experimental section for 2 min at 37 °C using 10 μ g of undepleted rod-outer-segment membranes. PDE γ was added at 4 μ g. Treated PDE γ was preincubated (4 μ g) with 4 μ g of carboxypeptidase-Y for 3 min at 20 °C. The reaction was stopped by adding 1 mM (final concn.)-PMSF. '% of control' is the percentage of [32 P]P $_i$ released compared with a control sample with no PDE γ added. Results are means for triplicate experiments.

Sample	Activity (% of control)
Control	100
+ PDE γ	42
+ PDE γ (treated)	37

Fig. 1. Western blots of depleted rod-outer-segment membranes

Membranes were treated as described in the Experimental section. Abbreviations: dep, depleted rod-outer-segment membranes; EDTA, depleted membranes washed four times with 1 mM-EDTA buffer [10 mM-Tris/HCl(pH 7.4)/1 mM-DTE/1 mM-EDTA]; Urea, EDTA-washed membranes washed once with 5 M-urea. The EDTA-washed membranes contained 0.74 μ g of rhodopsin, 0.0027 μ g of PDE γ and 0.0024 μ g of PDE α/β per ml. Arrows indicate the positions of corresponding proteins. Antisera, utilized at 1:100, are indicated at the bottom: α PDE γ -6, anti-PDE γ -6; α T α C, anti-T α C; α PDE α NT, anti-PDE α NT; α T β C, anti-T β C; α SAG-2, anti-SAG-2. Antisera are described in the Experimental section.

Fig. 2. Western blots of EDTA-depleted rod-outer-segment membranes treated with various proteinases

Treatment of membrane preparations were as described in the Experimental section. Abbreviations etc.: Carboxy Y, carboxypeptidase-Y-treated membranes. Numbers indicate periods of incubation (min) with corresponding proteinases. C, control untreated EDTA-depleted membranes. R, undepleted rod-outer-segment membranes ($\approx 10 \mu$ g total protein). Arrows indicate positions of PDE α and PDE γ as revealed by using anti-PDE α NT and anti-PDE γ -6 antisera at 1:100 and 125 I-Protein A.

low-affinity, PDE γ subunit results in a soluble PDE γ -T α -GTP[S] complex [8,9], full activation by higher concentrations of T α -GTP[S] is reported to leave the PDE $\alpha/\beta/\gamma$ membrane-bound [8,9]. We have used the EDTA-treated membranes as a source of intrinsic PDE in order to determine the effects of T α on membrane-associated PDE activity. In order to obtain a membrane-bound PDE α/β which was partially free of PDE γ , various proteolytic enzymes were used (Fig. 2). For soluble PDE, short-

term trypsin hydrolysis has been employed to obtain a fully activated PDE which is free of PDE γ [6]. However, as indicated in Fig. 2, exposure of the EDTA-washed membranes to trypsin, even for 1 min, resulted in full loss of PDE α/β and PDE γ from the membrane. Cathepsin C, an N-terminal-specific dipeptidase used as a control, did not remove PDE γ from the membrane preparation. However, carboxypeptidase-Y, a C-terminal-specific proteinase, removed the inhibitory activity of PDE γ

Fig. 3. Western blots of control and proteinase-treated depleted-rod outer-segment membranes

Each lane contained 33 μ l of membrane preparation containing 24.42 μ g of rhodopsin. All lanes contained 0.08 μ g of PDE α/β . Results show proteins on SDS/7.5% (w/v)-PAGE revealed with 125 I-protein A. Bands were allowed to react with a mixture of anti-PDE α NT (top band) and anti-PDE γ -6 (bottom band). Antisera were used at 1:100 each. R, undepleted bovine rod outer-segment preparation (≈ 10 μ g total protein). Lanes were: 1, control membranes alone; PDE γ = 0.08 μ g; 2, control membranes + 0.35 μ g of added PDE γ ; total 0.43 μ g; 3, control membranes + 0.70 μ g of added PDE γ ; total 0.78 μ g; 4, carboxypeptidase-Y-treated membranes alone; no detectable PDE γ ; 5, carboxypeptidase-Y-treated membranes + 0.35 μ g of added PDE γ ; total 0.35 μ g; 6, carboxypeptidase-Y-treated membranes + 0.70 μ g of added PDE γ ; total 0.70 μ g; 7, cathepsin C-treated membranes alone; PDE γ 0.08 μ g; 8, cathepsin C-treated membranes + 0.35 μ g of added PDE γ ; total 0.43 μ g; 9, cathepsin C-treated membranes + 0.70 μ g of added PDE γ ; total 0.78 μ g.

from the membranes without altering PDE α/β content. These membranes were used to determine the effects of proteolysis and/or re-addition of retinal proteins upon PDE enzyme activity.

The results of removal and re-addition of PDE γ to carboxypeptidase-Y-treated membranes are illustrated in Fig. 3 and Table 3. In Table 3 the effect of proteolysis and re-addition of purified PDE γ on PDE activity is summarized. Removal of PDE γ by carboxypeptidase-Y results in activation of PDE. This enzyme can be re-inhibited by the addition of 0.35 μ g of PDE γ . As Fig. 3 shows (lanes 4, 5 and 6), carboxypeptidase-Y partially removed PDE γ (lane 4) and PDE γ could be re-added to these membrane preparations.

We have also determined the effects of adding T α -GTP[S] to the carboxypeptidase-Y-treated membranes with and without PDE γ . These results are summarized in Table 3 and Fig. 4. Control membranes (not proteinase treated) had low endogenous PDE activity which was not further inhibited by the addition of PDE γ . In contrast, the carboxypeptidase-Y-treated membranes had higher PDE activity which was fully inhibited by adding exogenous PDE γ . The PDE γ added was calculated to restore the membrane-bound PDE α/β :PDE γ ratio to the PDE α/β : γ_2 of the holoenzyme complex. The effects of PDE γ addition on membrane-bound PDE γ content was similar to that illustrated in Fig. 3 and Table 3. When T α -GTP[S] was added to approx. 2 μ M, the PDE activity of the control membranes (lanes 1–5) was greatly activated. This occurs even though PDE γ is not

Table 3. Effect of T α on PDE activity of proteinase-treated depleted rod outer-segment membranes

EDTA-treated rod outer segments (10 μ l) were prepared as described in the Experimental section. The rhodopsin content of membranes (10 μ l) was 28 μ g/assay; that of PDE α/β was 0.128 μ g/assay; the PDE γ content of untreated membranes was 0.096 μ g/assay. Carboxypeptidase-Y-treated membranes contained no detectable PDE γ (as measured with anti-PDE γ -6 antisera), unless additional PDE γ was added. Control membranes are those which had no proteinase treatment. Membranes treated with carboxypeptidase-Y or cathepsin C were pretreated with proteinases as described in the Experimental section. As illustrated in Fig. 2, carboxypeptidase-Y-treated membranes contained no anti-PDE γ -6-reactive material, and the amounts of PDE α/β and of rhodopsin were identical with those in the control group. PDE γ final concentrations were as in Fig. 3. Additions were made as follows: PDE γ , 0.28 μ g; T α -GTP[S], 2.6 μ g (≈ 2 μ M); T $\alpha\beta\gamma$, T α = 1.6 μ g, T β = 3.2 μ g. Treated PDE γ was treated as in Table 1; 0.28 μ g was added/assay. PDE activity was measured using 40 μ M-cyclic [3 H]GMP. 'Max.' means the total radioactivity in assay. Results are means $\pm$ S.D. for four samples. Backgrounds were: expt. 1, 15.6%; expt. 2, 13.8%. These have been subtracted from the activity values.

Sample	PDE activity (c.p.m.)	
	Expt. 1	Expt. 2
Max.	32207 $\pm$ 2204	74906 $\pm$ 2853
Control membranes		
Alone	5403 $\pm$ 578	3824 $\pm$ 523
+ PDE γ	5762 $\pm$ 259	5178 $\pm$ 596
+ T α -GTP[S]	19089 $\pm$ 2181	39250 $\pm$ 1686
+ PDE γ (treated)	—	5734 $\pm$ 617
+ T $\alpha\beta\gamma$	—	12342 $\pm$ 166
Carboxypeptidase-Y-treated membranes		
Alone	12390 $\pm$ 1975	8998 $\pm$ 1579
+ PDE γ	5997 $\pm$ 575	3994 $\pm$ 708
+ T α -GTP[S]	18697 $\pm$ 1349	25918 $\pm$ 451
+ PDE γ (treated)	10186 $\pm$ 876	7854 $\pm$ 246
+ T $\alpha\beta\gamma$	—	12726 $\pm$ 1095

removed from the membrane, and it resulted in binding of the T α -GTP[S] to the membrane (Fig. 4b, lane 3) [9]. When T α -GTP[S] was added to carboxypeptidase-Y-treated membranes (lanes 6–7), the PDE activity was also activated (lane 8). This is most likely due to the presence of the proteolytic fragment of PDE γ (not detected in our assay) which has a T α -binding site and a possible alternate PDE α/β -binding site. The results of deletion mutation of PDE γ have suggested that such an alternative site exists [24]. As Table 1 shows, pretreatment of the exogenous PDE γ with carboxypeptidase-Y rendered it incapable of inhibiting the membrane-bound PDE α/β . When a T $\alpha/\beta/\gamma$ preparation was added to the control or treated membranes, PDE activation was also observed, although not to the level noted with T α alone (lanes 5 and 10, T $\alpha/\beta/\gamma$; lanes 3 and 8, T α alone). This indicates that T α can activate membrane-associated PDE even in the presence of T β/γ .

DISCUSSION

It has recently been reported that there are two PDE γ subunits per PDE α/β [5]. At low levels of T α -GTP, a low-affinity PDE γ is released from the membrane as a soluble T α -PDE γ complex *in vitro* [8,9]. This results in only 5–15% activation of the PDE enzyme in bovine samples, but in 50% activation of frog PDE [8,9]. This indicates that the control of PDE α/β enzyme activity by the PDE γ inhibition is species-specific. Full activation of PDE

Fig. 4. Western blots of control and carboxypeptidase-Y-treated depleted rod-outer-segment membranes

Experiments are described in Table 1. Each lane contained 40 μ l of depleted rod-outer-segment membranes which had 56 μ g of rhodopsin, 0.256 μ g of PDE α/β and 0.192 μ g of PDE γ (if control). To test lanes were added the following: PDE γ , 0.56 μ g; PDE γ -treated, 0.56 μ g (treated with carboxypeptidase Y as in Table 1); T α -GTP[S], 5.2 μ g; or 3.2 μ g of T $\alpha\beta\gamma$ and 6.4 μ g of T β . Antisera were used at 1:100. (a) Western blot treated with anti-PDE α NT and anti-T β C. (b) Western blot treated with anti-T α C. Lanes in (a) and (b) were: 1–5, control membranes; 6–10, carboxypeptidase-Y-treated membranes. Lanes: 1, control membranes; 2, control+PDE γ ; 3, control+T α -GTP[S]; 4, control+PDE γ -treated; 5, control+T $\alpha\beta\gamma$; 6, treated membranes; 7, treated+PDE γ ; 8, treated+T α -GTP[S]; 9, treated+PDE γ (treated); 10, treated+T $\alpha\beta\gamma$. R, undepleted rod-outer-segment membranes (10 μ g total protein).

required greater than 4 μ M-T α -GTP[S], and this did not result in the release of additional soluble PDE γ . This is presumably due to binding of T α -GTP[S] to the membranes without release of PDE γ . We have analysed the effects of high T α -GTP[S] on PDE activity of membrane-associated intrinsic PDE. This PDE remains tightly bound to the membrane after extensive washing in 1 mM-EDTA. We estimate that the PDE α/β has one PDE γ bound at this point. The exogenous T α -GTP[S] binds directly to the membranes, activates the PDE and does not result in further release of a soluble PDE γ -T α complex [9]. This occurs even when T $\beta\gamma$ is present, although to a lesser extent.

The membrane-bound PDE α/β does not require intact PDE γ

in order to remain associated with the membrane. Once PDE γ is treated with carboxypeptidase-Y, the PDE α/β is activated. Inhibition can be restored by re-addition of exogenous PDE γ . Carboxypeptidase-Y treatment of membrane-associated PDE does not result in an altered K_m for cyclic GMP, as is noted with trypsin. Furthermore, whereas trypsin removed PDE $\alpha/\beta/\gamma$ from the membranes, inhibition by PDE γ is removed only by carboxypeptidase Y. The carboxypeptidase Y may remove the PDE γ inhibitory site for PDE α/β . Carboxypeptidase-Y-treated PDE γ does not inhibit either soluble or membrane-associated PDE α/β . When synthetic peptides are used to identify the PDE γ -binding site, the peptide corresponding to residues 80–87, the C-terminus, was the most effective inhibitor.

From these data a model is proposed [9] in which, at low levels of T α -GTP[S], one PDE γ is removed from the membrane-associated PDE complex. This results in (1) low initial activation of PDE, (2) a remaining membrane-associated PDE $\alpha/\beta/\gamma$, (3) a soluble PDE γ -T α -GTP[S] complex *in vitro*, and (4) release of a soluble PDE. It has recently been reported that soluble PDE γ is found associated with either T α -GTP or T α -GDP [25]. Presumably the PDE γ -T α -GTP complex is altered via the T α GTPase to a PDE γ -T α -GDP complex. Removal of the PDE γ requires T β/γ [25]. Presumably the PDE γ then re-binds to PDE.

In contrast, full activation of PDE appears to require higher levels of T α -GTP[S], does not result in a soluble complex, and requires direct binding of T α -GTP[S] to the membrane without removal of PDE γ from the membrane. The exact mechanism by which this occurs, or the identity of the binding sites involved, remains to be elucidated. Furthermore, since multiple rod-outer-segment PDEs have been reported to exist [26], the role of these soluble and membrane-bound forms must be determined.

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