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

Jess M. CUNNICK, Daniel HURT, Brenda OPPERT, Kazuko SAKAMOTO and Dolores J. TAKEMOTO*
Department of Biochemistry, Kansas State University, Willard Hall, Manhattan, KS 66506, U.S.A.

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 (PDEα/β) and the α-subunit of the retinal G (guanine-nucleotide-binding)-protein transducin α (Tα). We have previously reported that the PDEγ binds to Tα at residue nos. 24–45 [Morrison, Rider & Takemoto (1987) FEBS Lett. 222, 266–270]. In vitro this results in inhibition of Tα GTP/GDP exchange [Morrison, Cunnick, Oppert & Takemoto (1989) J. Biol. Chem. 264, 11671–11681]. We now report that the inhibitory region of PDEγ for PDEα/β occurs at PDEγ residues 54–87. This binding results in inhibition of either trypsin-solubilized or membrane-bound PDEα/β. PDEγ which has been treated with carboxypeptidase Y, removing the C-terminus, does not inhibit PDEα/β, but does inhibit Tα GTP/GDP exchange. Inhibition by PDEγ can be removed by Tα-guanosine 5'-[γ-thio]triphosphate (GTP[S]) addition to membranes. This results in a displacement of PDEγ, but not in removal of this subunit from the membrane [Whalen, Bitsensky & Takemoto (1990) Biochem. J. 265, 655–658]. These results suggest that low levels of Tα–GTP[S] can result in displacement of PDEγ from the membrane in vitro as a GTP[S]–Tα–PDEγ complex. Further activation by high levels of Tα–GTP[S] occurs by displacement of PDEγ from its inhibitory site on PDEα/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 αβγ complex [4,5]. M₃ values for the α, β and γ subunits determined by SDS/PAGE are 88,000, 84,000, and 11,000 respectively [4]. The PDEγ subunit (PDEγ) inhibits PDE catalytic activity through a decrease in Vₘₐₓ, leaving the Kₘ unaltered [6,7]. The full activation of PDEα/β requires the removal by transducin α (Tα)–GTP of both PDEγ subunits [8]. Each PDEγ binds with a different affinity, and in the bovine retina, removal of the low-affinity PDEγ results in a 5–17% activation of the PDEα/β [8,9]. The second high-affinity PDEγ is removed at high concentrations of Tα–GTP and results in full activation of PDEα/β [8,9]. Evidence suggests that Tα–GTP can activate the PDEα/β by directly interacting with PDEγ to facilitate its removal from PDEα/β [7,8]. Removal of the low-affinity PDEγ requires lower amounts of Tα–GTP and results in the release of a soluble guanosine 5'-[γ-thio]triphosphate (GTP[S])–Tα–PDEγ complex in vitro [8,9]. This may or may not occur in vivo. At high levels of Tα–GTP[S] full activation of PDEα/β occurs, but this does not result in the release of a GTP[S]–Tα–PDEγ complex [8,9]. Presumably Tα, PDEα/β and PDEγ 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 PDEα/β enzyme which remained membrane-bound. Partial trypsin digestion of PDEα/β leads to the degradation of PDEγ and subsequent activation of the PDE [6]. However, this treatment results in the irreversible removal of PDEα/β from the membrane [10] and in an altered Kₘ as well as the characteristic increase in Vₘₐₓ [6,7]. We have used carboxypeptidase Y to obtain a membrane-bound PDEα/β which is inhibited by PDEγ and reactivated by Tα–GTP[S]. This has been used to study the inactivation process and to identify the binding sites of PDEγ for Tα and for PDEα/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-Manheim. [γ-32P]GTP (50 Ci/mmol), and [35S]GTP[S] (1200 Ci/mmol), were from NEN/dupont. Cyclic [8-3H]GMP (19 Ci/mmol) was from ICN Radiochemicals. Carrier-free 115I was from Amersham International. The t-butoxycarbonyl-amino acids and their resins were from Veba 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 Sequenol 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 death. 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.
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. 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 mM-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 PDEα/β/γ 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 PDEα/β/γ was further purified by concentrating the eluate with Aqueacid 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 antiserum to PDEα and to PDE-γ.

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. 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 antiserum to Tα and Tβ.

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 α/β/γ for membrane reconstitution experiments. In some cases, this membrane preparation was washed once with 5 mM-urea. Soluble PDEγ 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-micro fibre 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]P₁ released from the γ-phosphate of GTP was determined by liquid-scintillation counting. In some experiments, peptides or PDEγ 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 (150000 c.p.m./tube; specific radioactivity 19 Ci/mmol) in a final volume of 400 μl. Reactions took place at 30 °C. Purified PDEγ 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 Tα/γ, or purified PDEγ 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 PDEγ 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 PDEγ, Tα-GTP[S] or Tα/γ 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 PDEγ 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 unspayed lanes were scraped off and the peptide was eluted with 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
Table 1. Effect of PDEγ peptides on PDE activity

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peptide sequence</th>
<th>Peptide added (µg)</th>
<th>Activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin-activated</td>
<td>–</td>
<td>–</td>
<td>100 100 100</td>
</tr>
<tr>
<td>Inhibited</td>
<td>–</td>
<td>–</td>
<td>63 55 33</td>
</tr>
<tr>
<td>+Py1</td>
<td>1-14</td>
<td>–</td>
<td>100 110</td>
</tr>
<tr>
<td>+Py2</td>
<td>16-30</td>
<td>–</td>
<td>90</td>
</tr>
<tr>
<td>+Py3</td>
<td>31-45</td>
<td>–</td>
<td>107 101</td>
</tr>
<tr>
<td>+Py4</td>
<td>46-60</td>
<td>–</td>
<td>98 112</td>
</tr>
<tr>
<td>+Py5</td>
<td>61-74</td>
<td>–</td>
<td>70</td>
</tr>
<tr>
<td>+Py6</td>
<td>73-87</td>
<td>–</td>
<td>29</td>
</tr>
<tr>
<td>+Py7</td>
<td>54-60</td>
<td>–</td>
<td>70 101</td>
</tr>
<tr>
<td>+Py8</td>
<td>61-72</td>
<td>–</td>
<td>98 132</td>
</tr>
<tr>
<td>+Py9</td>
<td>73-79</td>
<td>–</td>
<td>112 87</td>
</tr>
<tr>
<td>+PDEγ-1</td>
<td>80-87</td>
<td>–</td>
<td>101 67</td>
</tr>
<tr>
<td>PDEγ-6</td>
<td>–</td>
<td>–</td>
<td>27 28</td>
</tr>
<tr>
<td>PDEγ (treated)</td>
<td>–</td>
<td>–</td>
<td>107 102</td>
</tr>
</tbody>
</table>

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 µl 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 µl of water. Portions (10 µl) 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: PDEγ-6, against residues 73-87 of PDEγ; SAg-2 against residues 225-239 of S-antigen; TaC, against residues 326-350 of Ta; TβC, against residues 326-340 of Tβ; PDE-αNT, against residues 1-15 of PDEα.

Quantification

Protein concentrations were determined by the method of Bradford [19] or Lowry et al. [20] using BSA as a standard. SDS/PAGE was described as [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 PDEγ [22]. These peptides were designated PDEγ-1–5.

PDE activity was measured as described [14], using 40 µM-cyclic GMP at 30 °C for 5 min in a final volume of 400 µl. The trypsin-activated and inhibited PDEγs were prepared as described in the Experimental section. Quantities, in 400 µl (final volume) of assay mixture, were 0.32 µg of trypsin-activated PDE or 0.4 µg of inhibited PDE. PDEγ was prepared as described in the Experimental section and 0.35 µg was added per assay. The PDEγ (treated) sample was identical with PDEγ, but was preincubated for 5 min at 30 °C with 0.1 µg of carboxypeptidase-Y. 0.5 mM-PMSF was added to stop proteolysis. This PDEγ preparation was used at 0.35 µg/assay. Peptides (Py1 etc.) were added to the assay before the substrate. Under 'Peptide sequence', residue numbers refer to the amino acid sequence of bovine PDEγ [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 µg/assay. Results are means for four samples.

Table 2. Effect of PDEγ on Ta GTaPase activity

The GTaPase activity of Ta was measured as described in the Experimental section for 2 min at 37 °C using 10 µg of undepleted rod-outter-segment membranes. PDEγ was added at 4 µg. Treated PDEγ was preincubated (4 µg) with 4 µg 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 [35S]GTPγS released compared with a control sample with no PDEγ added. Results are means for triplicate experiments.
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 μg of rhodopsin, 0.0027 μg of PDEγ and 0.0024 μg of PDEα/β per ml. Arrows indicate the positions of corresponding proteins. Antisera, utilized at 1:100, are indicated at the bottom: αPDEγ-6, anti-PDEγ-6; αTaC, anti-TaC; αPDEαNT, anti-PDEαNT; αTβC, anti-TβC; αSAg-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 (≈ 10 μg total protein). Arrows indicate positions of PDEα and PDEγ as revealed by using anti-PDEαNT and anti-PDEγ-6 antisera at 1:100 and 125I-Protein A.

low-affinity, PDEγ subunit results in a soluble PDEγ–Ta–GTP[S] complex [8,9], full activation by higher concentrations of Ta–GTP[S] is reported to leave the PDEα/β/γ membrane-bound [8,9]. We have used the EDTA-treated membranes as a source of intrinsic PDE in order to determine the effects of Ta on membrane-associated PDE activity. In order to obtain a membrane-bound PDEα/β which was partially free of PDEγ, 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 PDEγ [6]. However, as indicated in Fig. 2, exposure of the EDTA-washed membranes to trypsin, even for 1 min, resulted in full loss of PDEα/β and PDEγ from the membrane. Cathepsin C, an N-terminal-specific dipeptidase used as a control, did not remove PDEγ from the membrane preparation. However, carboxypeptidase-Y, a C-terminal-specific proteinase, removed the inhibitory activity of PDEγ.
Fig. 3. Western blots of control and proteinase-treated depleted-rod outer-segment membranes

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

from the membranes without altering PDEα/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 PDEγ 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 PDEγ on PDE activity is summarized. Removal of PDEγ by carboxypeptidase-Y results in activation of PDE. This enzyme can be re-inhibited by the addition of 0.35 μg of PDEγ. As Fig. 3 shows (lanes 4, 5 and 6), carboxypeptidase-Y partially removed PDEγ (lane 4) and PDEγ could be re-added to these membrane preparations.

We have also determined the effects of adding Ta-γ-GTP[S] to the carboxypeptidase-Y-treated membranes with and without PDEγ. 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 PDEγ. In contrast, the carboxypeptidase-Y-treated membranes had higher PDE activity which was fully inhibited by adding exogenous PDEγ. The PDEγ added was calculated to restore the membrane-bound PDEα/β:PDEγ ratio to the PDEα/βγ2 of the holoenzyme complex. The effects of PDEγ addition on membrane-bound PDEγ content was similar to that illustrated in Fig. 3 and Table 3. When Ta-γ-GTP[S] was added to approx. 2 μm, the PDE activity of the control membranes (lanes 1–5) was greatly activated. This occurs even though PDEγ is not removed from the membrane, and it resulted in binding of the Ta-γ-GTP[S] to the membrane (Fig. 4b, lane 3) [9]. When Ta-γ-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 PDEγ (not detected in our assay) which has a Ta-β-binding site and a possible alternate PDEα/β-binding site. The results of deletion mutation of PDEγ have suggested that such an alternative site exists [24]. As Table 1 shows, pretreatment of the exogenous PDEγ with carboxypeptidase-Y rendered it incapable of inhibiting the membrane-bound PDEα/β. When a Ta-β/γ preparation was added to the control or treated membranes, PDE activation was also observed, although not to the level noted with Ta alone (lanes 5 and 10, Ta-β/γ; lanes 3 and 8, Ta alone). This indicates that Ta can activate membrane-associated PDE even in the presence of Tβ/γ.

**DISCUSSION**

It has recently been reported that there are two PDEγ subunits per PDEα/β [5]. At low levels of Ta-γ-GTP, a low-affinity PDEγ is released from the membrane as a soluble Ta-γ-PDEγ 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 PDEα/β enzyme activity by the PDEγ inhibition is species-specific. Full activation of PDE
required greater than 4 μM-Tα-GTP[S], and this did not result in the release of additional soluble PDEγ. This is presumably due to binding of Tα-GTP[S] to the membranes without release of PDEγ. We have analysed the effects of high Tα-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 PDEα/β has one PDEγ bound at this point. The exogenous Tα-GTP[S] binds directly to the membranes, activates the PDE and does not result in further release of a soluble PDEγ-Tα complex [9]. This occurs even when Tβγ is present, although to a lesser extent.

The membrane-bound PDEα/β does not require intact PDEγ in order to remain associated with the membrane. Once PDEγ is treated with carboxypeptidase-Y, the PDEα/β is activated. Inhibition can be restored by re-addition of exogenous PDEγ. 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 PDEα/βγ from the membranes, inhibition by PDEγ is removed only by carboxypeptidase Y. The carboxypeptidase Y may remove the PDEγ inhibitory site for PDEα/β. Carboxypeptidase-Y-treated PDEγ does not inhibit either soluble or membrane-associated PDEα/β.

When synthetic peptides are used to identify the PDEγ-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 Tα–GTP[S], one PDEγ is removed from the membrane-associated PDE complex. This results in (1) low initial activation of PDE, (2) a remaining membrane-associated PDEα/βγ, (3) a soluble PDEγ-Tα–GTP[S] complex in vitro, and (4) release of a soluble PDE. It has recently been reported that soluble PDEγ is found associated with either Tα–GTP or Tα–GDP [25]. Presumably the PDEγ-Tα–GTP complex is altered via the Tα GTPase to a PDEγ-Tα–GDP complex. Removal of the PDEγ requires Tβγ [25]. Presumably the PDEγ then re-binds to PDE.

In contrast, full activation of PDE appears to require higher levels of Tα–GTP[S], does not result in a soluble complex, and requires direct binding of Tα–GTP[S] to the membrane without removal of PDEγ 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-outersegment PDEs have been reported to exist [26], the role of these soluble and membrane-bound forms must be determined.

This work was supported by National Institutes of Health grant EY06490 (to D.J.T.), and by a grant from the American Heart Association, Kansas Affiliate. K.S. is a Scholar of the Wesley Scholar Program, Wichita, KS, U.S.A.

REFERENCES

Binding of retinal phosphodiesterase subunit γ to subunits α/β and transducin


Received 30 March 1990; accepted 3 May 1990