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Two-site high-affinity interaction between inhibitory and catalytic subunits of rod cyclic GMP phosphodiesterase

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Light-activated cyclic GMP-phosphodiesterase (PDE) is the key effector enzyme of vertebrate photoreceptor cells which regulates the level of the internal transmitter cyclic GMP. PDE consists of catalytic Pα and Pβ subunits, and two copies of inhibitory Pγ subunit. The two Pγ subunits block the enzyme's activity in the dark and are removed by the α-subunit of transducin (α_t) upon light-activation of photoreceptor cells. Here we have examined the role of various regions of Pγ, the *N*-terminal, the central cationic and the *C*-terminal regions, in interaction with the catalytic subunits of PDE. *N*-Terminal truncation of Pγ (12–87-Pγ) did not change the potency of PDE inhibition, and thus we conclude that the Pγ *N*-terminal region is not critical for Pγ–Pαβ interaction. The central region, 24–46-Pγ, participates in interaction with the catalytic Pαβ subunits. A synthetic peptide corresponding to this site inhibited ~ 50% of trypsin-activated PDE (tPDE) ($K_i \sim 15$ μM) and competed with Pγ for inhibition of tPDE. We demonstrated, by using h.p.l.c. gel filtration, that ^{125}I-Tyr-24–46-Pγ peptide bound with high affinity to tPDE, but not to $P\alpha\beta\gamma_2$. The *C*-terminal region of 46–87-Pγ was found to be the major region involved in inhibition of PDE. It fully inhibited tPDE with a K_i of ~ 0.8 μM. It also bound to tPDE, but not $P\alpha\beta\gamma_2$, in h.p.l.c. gel-filtration experiments. In addition, Pγ was cross-linked by *p*-phenylenedimaleimide to both Pα and Pβ, as was shown by using subunit-specific anti-Pα, -Pβ and -Pγ antibodies. Cys^{68} of Pγ, which presumably partipicates in cross-linking, is located near the Pγ *C*-terminus. These data provide evidence for two regions of Pγ that interact with, and inhibit, Pαβ. The central region, 24–46-Pγ, is important in binding, but inhibits PDE only weakly, whereas the *C*-terminal region is most important for PDE inhibition. These results help to explain the well-known fact that Pγ trypsin-activation and *C*-terminal truncation both lead to PDE activation. Furthermore, our findings on the mechanism of PDE inhibition of Pγ are relevant for understanding the mechanism of PDE activation by transducin.

INTRODUCTION

Photoreceptor cyclic GMP phosphodiesterase (PDE) is the effector enzyme in the visual signalling cascade of proteins in the vertebrate photoreceptor cell. Interaction between photoexcited rhodopsin and a GTP-binding protein, G_t, causes GDP–GTP exchange on the α_t subunit. The active complex, α_tGTP, dissociates from the $\beta\gamma_t$ subunit and the photoreceptor membrane and activates PDE. PDE activation leads to a decrease in the intracellular concentration of cyclic GMP, subsequent closure of cyclic GMP-dependent cation channels in the photoreceptor plasma membrane and hyperpolarization of the membrane [for reviews, see Stryer (1986), Hurley (1987), Liebman *et al.* (1987), Chabre & Deterre (1989) and McNaughton (1990)].

The PDE is composed of catalytic Pα (88 kDa) and Pβ (85 kDa) subunits, and two copies of inhibitory Pγ subunits, which block enzyme activity in the dark (Baehr *et al.*, 1979; Hurley & Stryer, 1982; Deterre *et al.*, 1988). Evidence suggests that α_tGTP activates PDE through direct binding to Pγ in the holoenzyme form, $P\alpha\beta\gamma_2$, followed by dissociation of the complex α_tGTP–Pγ from Pαβ (Pαβγ) (Deterre *et al.*, 1986; Wensel & Stryer, 1986; Fung & Griswold-Prenner, 1989; Wensel & Stryer, 1990; Yamazaki *et al.*, 1990).

α_tGTP may interact with Pαβ (Hingorani *et al.*, 1988), and one report suggests that α_tGTP–Pαβ interaction is the major event in the PDE activation process (Sitaramayya *et al.*, 1986). In addition, other data (Kroll *et al.*, 1989) are consistent with the hypothesis that Pγ does not completely dissociate from the enzyme and that de-activation of PDE includes a direct interaction of Pαβ with α_tGDP.

PDE can also be activated by limited tryptic proteolysis of Pγ (Hurley & Stryer, 1982), by polycationic proteins such as histone, protamine and polylysine (Miki *et al.*, 1975) or by *C*-terminal truncation (Lipkin *et al.*, 1988; Brown & Stryer, 1989). Studies of the mechanism of PDE inhibition by Pγ are key to understanding PDE activation of transducin.

The elucidation of the primary structures of all PDE subunits (Ovchinnikov *et al.*, 1986, 1987; Lipkin *et al.*, 1990*a*,*b*) opens up the possibility of a detailed study of intramolecular PDE interactions and its activation by G_t. Here we demonstrate that two regions, namely 24–46-Pγ and 46–87-Pγ, bind Pαβ and inhibit trypsin activated PDE (tPDE) activity.

EXPERIMENTAL

Materials

Cyclic GMP was purchased from Boehringer Mannheim. Tosylphenylalanylchloromethane ('TPCK')-treated trypsin and soybean trypsin inhibitor were obtained from Worthington. Histone from calf thymus (type II-A) was a product of Sigma. Protein A–Sepharose and Blue Sepharose SL-6B were products of Pharmacia. Na^{125}I, ^{125}I-labelled Protein A and glutaraldehyde (grade II; 25%, w/v) were from ICN Biomedicals. All other chemicals were from Sigma or Fisher.

Abbreviations used: PDE, photoreceptor cyclic GMP phosphodiesterase; Pα, Pβ and Pγ, subunits of phosphodiesterase; tPDE, trypsin-activated phosphodiesterase; α_t and $\beta\gamma_t$, subunits of the photoreceptor guanine-nucleotide-binding protein transducin; ROS, rod outer segment; GTP[S], guanosine 5'-[γ-thio]triphosphate; PDM, *p*-phenylenedimaleimide; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; IC_{50}, concentration causing 50% of maximum inhibition.

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Preparation of PDE, tPDE, Pγ and fusion protein

Bovine rod-outer-segment (ROS) membranes were prepared by the method of Papermaster & Dreyer (1974) with some modifications (Mazzoni *et al.*, 1991). PDE was extracted from bleached ROS membranes as described previously (Baehr *et al.*, 1979). The PDE-containing extract was concentrated by ultrafiltration using a YM-30 membrane (Amicon), and PDE was purified by ion-exchange h.p.l.c. on a Synchropak AX-300 column (10 cm × 0.41 cm) (SynChrom) The column was equilibrated with buffer A [20 mM-Hepes (pH 7.2)/1 mM-$MgCl_2$/100 mM-Na_2SO_4/1 mM-dithiothreitol (DTT)/0.1 mM-phenylmethanesulphonyl fluoride (PMSF). Proteins were eluted with a Na_2SO_4 gradient (100–800 mM)]. The fraction containing PDE was concentrated by using a Centricon 30 filter microconcentrator and applied to an h.p.l.c. gel-filtration column (Spherogel-TSK 3000SW; 30 cm × 0.75 cm; Altex). PDE was eluted with buffer B [10 mM-Mops (pH 8.0)/1 mM-$MgCl_2$/100 mM-NaCl/1 mM-DTT). DTT was omitted from buffer B when PDE was subsequently used for the cross-linking experiments. PDE obtained by these procedures was 99% pure on the basis of densitometric scanning of Coomassie Blue-stained SDS/polyacrylamide gels.

tPDE was prepared by treatment of purified PDE (1 mg/ml) with tosylphenylalanylchloramethane-treated trypsin (50 μg/ml) for 20 min at +4 °C. The reaction was terminated by the addition of a 10-fold excess of soybean trypsin inhibitor. The PDE was separated from trypsin and trypsin inhibitor by h.p.l.c. using a Spherogel-TSK 3000SW column. The absence of Pγ in the tPDE preparation was confirmed by SDS/PAGE and Western-blot analysis with polyclonal anti-Pγ antibodies. PDE and tPDE were stored in 40% (v/v) glycerol at −20 °C.

The Pγ subunit was purified by reversed-phase h.p.l.c. on a C-4 (25 cm × 0.46 cm) column (Vydac) with a 20–100% gradient of acetonitrile in 0.1% trifluoroacetic acid (Ovchinnikov *et al.*, 1986). Pγ was eluted at approx. 45% acetonitrile and stored in this solution at +4 °C for 1 month without loss of functional activity. A fusion protein, consisting of the first 31 residues of lambda cII protein, the clotting-proteinase (Factor Xa) cleavage site (seven residues) and the Pγ sequence (87 residues) was expressed and purified as described by Brown & Stryer (1989).

Preparation of 12–87-Pγ and 46–87-Pγ polypeptides

We found that proteolysis of the fusion protein with Factor Xa (Brown & Stryer, 1989) formed native Pγ, as well as a smaller fragment corresponding to residues 12–87. The additional Factor Xa cleavage site was determined by amino-acid-sequence analysis, the first ten cycles of which gave the sequence SATRVMGGPV. Amino acid sequence analysis was performed by using a pulsed liquid-phase protein sequencer (ABI 477A; Applied Biosystems). 12–87-Pγ was separated by SDS/20% PAGE, electroblotted to a poly(vinylidene difluoride) membrane (Applied Biosystems) and extracted from the membrane by boiling the membrane strip for 10 min in buffer [60 mM-Tris/HCl (pH 6.8)/2% Nonidet P40/6 M-urea/2% β-mercaptoethanol]. The 46–87-Pγ peptide was obtained by extensive trypsin treatment of the fusion protein (1 mg of protein/ml; 50 μg of trypsin/ml; 5 h; +37 °C) and purified twice by reversed-phase h.p.l.c. on a C-4 (25 cm × 0.46 cm) column (Vydac). Prolonged proteolysis, double purification and the difference of the elution points (50% acetonitrile for 46–87-Pγ and 45% for Pγ and fusion protein) yielded a fraction containing 46–87-Pγ with no contamination by Pγ and fusion protein. Amino-acid-sequence analysis showed that it consisted of 66% 46–87-Pγ and 34% of 45–87-Pγ. The tPDE-bound portion (see below) of ^{125}I-46–87-Pγ was collected and further analysed by SDS/PAGE. Autoradiography of the gel revealed no trace of Pγ or fusion protein.

Peptide synthesis

Peptides corresponding to 24–46-Pγ (Ovchinnikov *et al.*, 1986), 21–31-Pα, 38–49-Pα and 37–49-Pβ (Lipkin *et al.*, 1990*b*) were synthesized by the solid-phase Merrifield method on an Applied Biosystems automated peptide synthesizer in the Protein Sequencing/Synthesis Laboratory at University of Illinois at Chicago. Their sequences are:

24–46-Pγ Ac-RKGPPKFKQRQTRQFKSKPPKK-NH_2

21–31-Pα Ac-KQYYNLRYRAK-NH_2

38–49-Pα GPREAAVDFSNY-NH_2

37–49-Pβ EDGCPEGCTSFRE-NH_2

For the purpose of ^{125}I-labelling, 24–46-*Pγ* was also synthesized with extra tyrosine residue at the *N*-terminus. Each peptide was purified by reversed-phase h.p.l.c. on a preparative Aquapore Octyl column (25 cm × 1 cm) (Applied Biosystems). The purity and chemical formula of each peptide were confirmed by fast-atom-bombardment m.s. at the University of Illinois at Chicago Mass Spectrometry Facility, analytical h.p.l.c. and amino acid analysis. Peptides were freeze-dried and stored desiccated at −20 °C until use.

^{125}I-labelling of the protein

Fusion protein and Tyr-24–46-Pγ were ^{125}I-labelled as described by Fraker & Speck (1978). Typical specific radioactivities were 2.5 mCi/μmol of fusion protein and 1.9 mCi/μmol of Tyr-24–46-Pγ. The ^{125}I-labelled 46–87-Pγ was obtained by trypsin treatment of ^{125}I-labelled fusion protein as described above.

Preparation of anti-(PDE subunit)-specific antibodies

In order to obtain anti-Pα and anti-Pβ specific antibodies, peptides from Pα and Pβ subunits with minimum identity were synthesized: 21–31-Pα, 38–49-Pα and 37–49-Pβ. Approx 1 μmol of each peptide was conjugated with 0.1 μmol of BSA in 0.1 M-sodium phosphate buffer, pH 7.5, with 0.05% glutaraldehyde. The reaction was quenched after incubation for 1 h at room temperature by the addition of an excess of ethanolamine. The peptide conjugates were injected under the skin behind the neck at multiple points into 8–10-week-old New Zealand White rabbits. Initially 0.5 mg of each conjugate was injected together with the RIBI (RIBI ImmunoChem Research) adjuvant system. Every 3 weeks rabbits were boosted with 0.2 mg of peptide conjugates. Usually four or five immunizations were necessary to obtain an adequate immune response. At 10 days after the last immunization the rabbits were bled. The antibodies were purified from serum on a Protein A–Sepharose column and stored in 40% (v/v) glycerol at −20 °C. Anti-1–15-Pα antibodies were provided by Dr. D. Takemoto (Kansas State University). Specific anti-Pγ antibodies were prepared by immunizing the rabbits with purified Pγ. The Pγ (100 μg/rabbit) was injected with RIBI adjuvant into the rabbits in the same manner as described above. Every 2 weeks, rabbits were boosted with 50 μg of the protein. At 10 days after the fourth immunization the rabbits were bled, and antibodies were purified on a Protein A–Sepharose column. The specificity of the antibodies was tested by Western immunoblotting [Fig. 1 and Fig. 9*e* (below)]. In order clearly to separate Pα and Pβ subunits, prolonged SDS/PAGE was run in a low-cross-linked gel (15% acrylamide/0.08% bisacrylamide) (Baehr *et al.*, 1979).

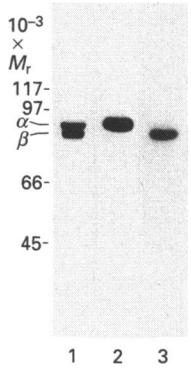

Fig. 1. Characterization of specific anti-(Pα subunit) and anti-(Pβ subunit) antibodies

Purified PDE was separated by prolonged electrophoresis on low-cross-linked SDS/15%-polyacrylamide gels by the method of Baehr *et al.* (1979) (lane 1, Coomassie Blue-stained SDS/PAGE gel) which maximizes separation between Pα and Pβ subunits. Pγ subunit was run out of the gel. After transfer to nitrocellulose paper, PDE was analysed with anti-1–15-Pα (lane 2) and anti-37–49-Pβ (lane 3) anti-peptide antisera as described in the Experimental section.

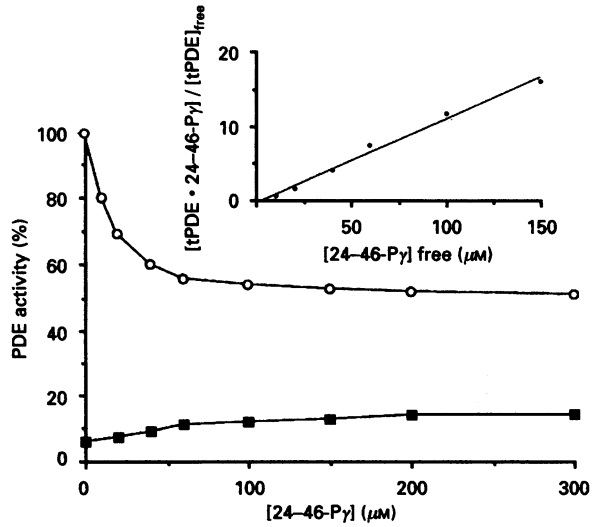

Fig. 2. Effect of synthetic peptide 24–46-Pγ on tPDE activity and basal PDE activity

The activities of tPDE (2.5 nM) (○) and PDE from ROS extract (2.5 nM) (■) were determined in the presence of increasing concentrations of 24–46-Pγ by the proton release assay as described in the Experimental section. The peptide was added before the initiation of the reaction by cyclic GMP. All activities are expressed as a percentage of tPDE activity. Linear transformation of the inhibition curve was made based on the assumption that the maximal inhibitory effect (50% inhibition) corresponds to 100% tPDE bound to 24–46-Pγ (r −0.985).

Detection of Pγ fragments binding to PDE using h.p.l.c. gel filtration

^{125}I-labelled fusion protein, Tyr-24–46-Pγ and 46–87-Pγ (300 pmol each) were preincubated with equimolar amounts of holo-PDE or tPDE for 15 min at room temperature in 100 μl of buffer B and injected into an Ultraspherogel 3000SW (30 cm × 0.75 cm) column (Beckman) equilibrated with the same buffer. Proteins were eluted from the column at a flow rate of 0.7 ml/min and radioactivity was measured by means of an M170 flow radioisotope detector (Beckman).

Chemical cross-linking of PDE with *p*-phenylenedimaleimide (PDM)

Before chemical cross-linking, PDE was transferred to buffer B (minus DTT) by using gel filtration on a Spherogel TSK 3000SW column. The PDE concentration was adjusted to 2.5 μM. PDM (1 mM) in dimethylformamide was added to a final concentration of 50 μM (the PDE/cross-linker ratio was 1:20). Cross-linking was allowed to proceed for 15 min at room temperature. The reaction was quenched by adding excess β-mercaptoethanol. PDE cross-linked products were analysed by SDS/PAGE and Western immunoblotting. The proteins were transferred to nitrocellulose (0.1 μM; Schleicher and Schuell) as described previously (Towbin *et al.*, 1979). The antibody–antigen complexes were detected with ^{125}I-Protein A (Mazzoni *et al.*, 1991). Immunoblots were exposed to Kodak X-AR 2 X-ray film.

PDE assay

The PDE activity was measured using the proton-evolution assay (Liebman & Evanczuk, 1982). The assay was carried out at room temperature in a final volume of 200 μl in 10 mM-Hepes buffer (pH 8.0)/100 mM-KCl, 2 mM-$MgCl_2$/1 mM-DTT. The PDE concentration in the assay was 2.5 nM. The reaction was initiated by the addition of cyclic GMP (4 mM final concn.). The pH was monitored with a pH microelectrode (Microelectrodes). Addition of the peptides to the assay mixture over the experimental range of concentrations did not change the buffering capacity of the system.

Analytical methods

Protein concentrations were determined by the Coomassie Blue binding method (Bradford, 1976) using γ-globulin as a standard. SDS/PAGE was performed by the method of Laemmli (1970). Densitometric scans of Coomassie Blue-stained gels were performed on an Ephortec Joyce–Loebl densitometer.

RESULTS

Role of Pγ regions in PDE binding and inhibition

In order to determine the role of various regions of Pγ in interaction with Pαβ we generated three different peptides: 12–87-Pγ, 24–46-Pγ and 46–87-Pγ and investigated both their ability to bind Pαβ and to affect PDE activity. A Pγ peptide, if it participates in interaction with Pαβ, is likely to be less efficient than native Pγ. Therefore we reasoned that the presence of Pγ in the enzyme sample would interfere with the effect of the fragments, and experiments were performed with tPDE.

We first studied the role of the *N*-terminal region by preparing an *N*-terminal truncation: 12–87-Pγ. We found that the 12–87-Pγ polypeptide at two concentrations (2 nM and 4 nM) was equipotent with Pγ in tPDE inhibition. This observation suggests that the *N*-terminal region plays no significant role in Pγ binding to Pαβ and PDE inhibition.

Effect of peptide 24–46-Pγ on tPDE and basal PDE activity

A peptide (24–46-Pγ) corresponding to the central polycationic region was chemically synthesized. Fig. 2 shows that this peptide inhibited tPDE activity in a dose-dependent manner with a K_i of ~ 15 μM. The inhibition curve reached saturation at a peptide concentration of 100 μM. The peptide 24–46-Pγ inhibited a maximum of 50% of tPDE activity, and higher doses up to 1 mM-peptide had no additional effect. Linear transformation of the inhibition curve showed one class of binding sites for 24–46-Pγ on tPDE (Fig. 2, inset).

The effect of 24–46-Pγ on basal PDE activity was determined. Rather than inhibiting basal PDE activity as well, the peptide actually stimulated basal PDE activity 2–2.5-fold (K_a ~ 40 μM),

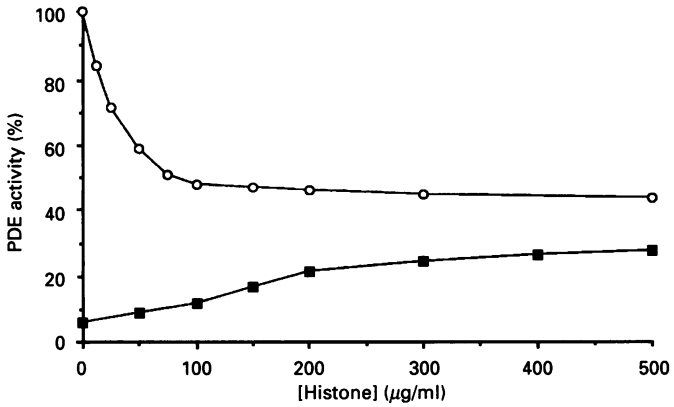

Fig. 3. Effect of histone on tPDE activity and basal PDE activity

The activities of tPDE (2.5 nM) (○) and PDE from ROS extract (2.5 nM) (■) were measured in the presence of increasing amounts of histone from calf thymus (type II-A). Histone was added before the initiation of the reaction by cyclic GMP. All activities are expressed as a percentage of tPDE activity.

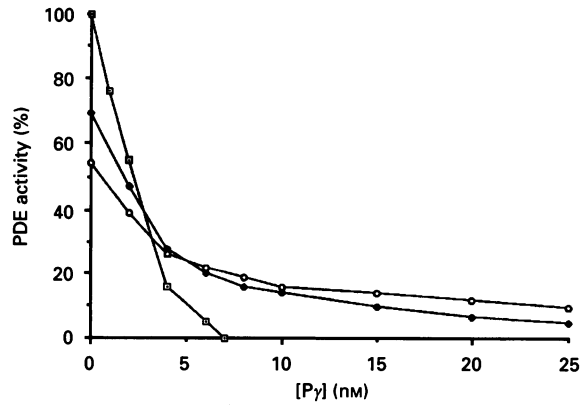

Fig. 4. Competition between Pγ and 24–46-Pγ for inhibition of tPDE

tPDE (2.5 nM) was titrated with Pγ in the absence of 24–46-Pγ (⊡) or in the presence of 20 μM- (●) or 100 μM- (○) 24–46-Pγ. The peptide was added before the start of the reaction with cyclic GMP. Then PDE activities were measured by the proton-release assay after the addition of increasing concentrations of Pγ. The 24–46-Pγ inhibited tPDE, but prevented Pγ from efficiently inhibiting the enzyme.

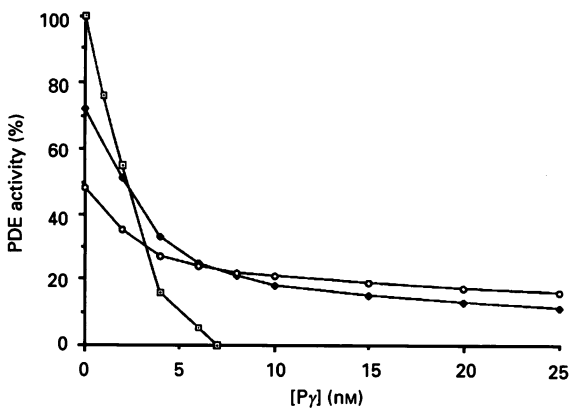

Fig. 5. Competition between Pγ and histone for the inhibition of tPDE

tPDE (2.5 nM) was titrated with Pγ in the absence of histone (⊡) or in the presence of 25 μg (●) or 100 μg (○) of histone/ml. Histone was added before the start of the reaction with cyclic GMP. Then PDE activities were measured by the proton-release assay after the addition of increasing concentrations of Pγ.

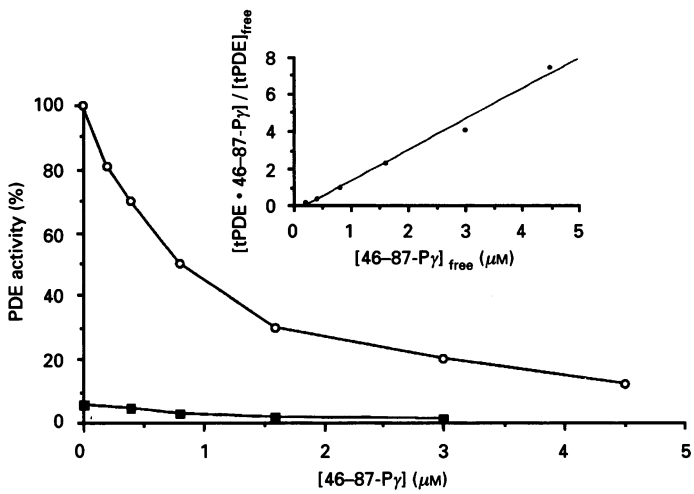

Fig. 6. Effect of 46–87-Pγ polypeptide on tPDE activity and basal PDE activity

The activities of tPDE (2.5 nM) (○) and PDE from ROS extract (2.5 nM) (■) were determined in the presence of increasing concentrations of 46–87-Pγ by the proton-release assay as described in the Experimental section. The polypeptide was added before the initiation of the reaction by cyclic GMP. All activities are expressed as a percentage of tPDE activity. Linear transformation of the inhibition curve gave a single slope (r −0.99).

in PDE preparations, obtained by hypo-osmotic extraction of ROS (Fig. 2).

It was of interest to compare the effects of 24–46-Pγ with those of polycationic proteins, which have also been found to activate basal PDE (Miki *et al.*, 1975). In the presence of histone the tPDE activity was inhibited [IC_{50} (concn. causing 50% of maximum inhibition) ~ 30 μg/ml or 2 μM] to a similar extent as was achieved by the Pγ peptide (Fig. 3). The effect of histone on basal PDE was greater than that of the peptide, an approx. 5-fold stimulation.

Competition of peptide 24–46-Pγ with Pγ for tPDE inhibition

If peptide 24–46-Pγ binds to Pαβ at the same site as Pγ, it should compete with Pγ for PDE inhibition. We observed such competition when tPDE was inhibited by Pγ in the presence of different concentrations of 24–46-Pγ. As seen in Fig. 4, Pγ inhibited 2.5 nM-tPDE with half-maximal inhibition occurring at Pγ concentrations of 2–3 nM, indicating that Pγ affinity for Pαβ is well below the nanomolar range, consistent with literature values (Wensel & Stryer, 1990). Pγ was considerably less effective in inhibiting tPDE in the presence of increasing concentrations of 24–46-Pγ. In the presence of 20 μM-peptide, a concentration near the peptide's K_i for tPDE, the K_i for Pγ inhibition was ~ 1.8 nM, whereas in the presence of 100 μM-peptide, the K_i was ~ 3.5 nM. Thus it appears that 24–46-Pγ partially blocks the site of Pγ and Pαβ interaction and prevents Pγ from effectively inhibiting catalytic activity. Histone, as well, competed with Pγ for tPDE inhibition (Fig. 5).

Effect of 46–87-Pγ polypeptide on tPDE and basal PDE activity

To investigate the role of the *C*-terminal region of Pγ, a tryptic fragment, 46–87-Pγ, was generated from the fusion protein. This fragment was much more potent at inhibiting tPDE than 24–46-Pγ (Fig. 6). It fully inhibited tPDE with a K_i of ~ 0.8 μM. Linear

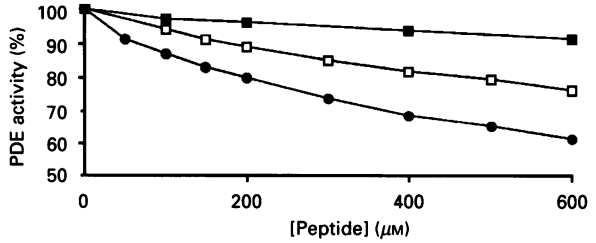

Fig. 7. Effects on tPDE activity of synthetic peptides corresponding to Pα and Pβ sequences

tPDE was assayed in the presence of increasing concentrations of peptides 21–31-Pα (●), 38–49-Pα (□) and 37–49-Pβ (■). The peptides were added to the indicated final concentration before the initiation of the reaction by cyclic GMP.

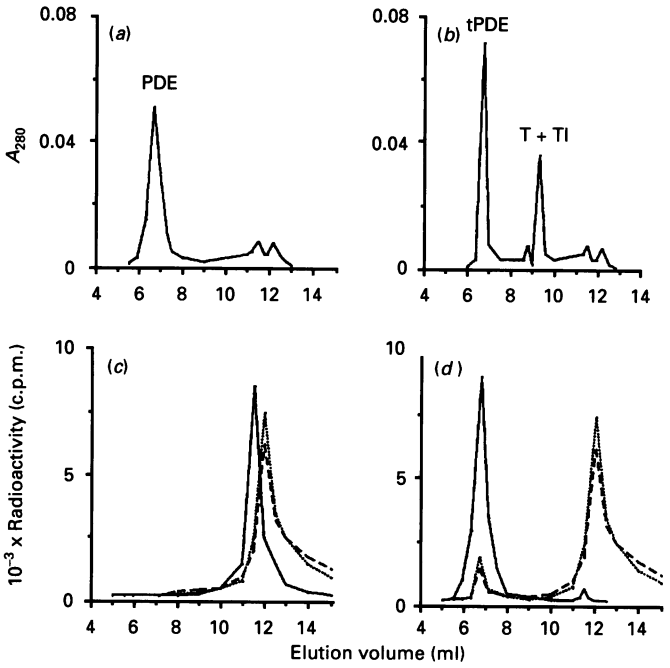

Fig. 8. Detection of binding of 24–26-Pγ and 46–87-Pγ to Pαβ using h.p.l.c. gel filtration

^{125}I-labelled fusion protein, ^{125}I-Tyr-24–46-Pγ or ^{125}I-46–87-Pγ were mixed with equimolar amounts of PDE (*a* and *c*) and tPDE (*b* and *d*) in buffer B. After 15 min incubation at room temperature, proteins were injected into an h.p.l.c. gel-filtration column and eluted with buffer B at a flow rate of 0.7 ml/min. (*a*) and (*b*) protein-elution profiles (A_{280}). (*c*) and (*d*) radioactivity profiles of ^{125}I-labelled fusion protein (——), ^{125}I-Tyr–24-46-Pγ (· · · · · ·), and ^{125}I-46–87-Pγ (– – – –). T + TI, trypsin + trypsin inhibitor.

transformation of this inhibition curve showed one class of binding sites for 46–87-Pγ on tPDE, as was observed for 24–46-Pγ. Furthermore, at the same range of concentration, 46–87-Pγ inhibited basal PDE activity (Fig. 6), an effect opposite to that of 24–26-Pγ.

Effects of Pαβ peptides on tPDE

Control experiments were performed with three different peptides from Pα and Pβ *N*-terminal regions. One of these peptides, 21–31-Pα, inhibited tPDE with low affinity (Fig. 7). At 600 μM-peptide, ~ 40% of tPDE activity was inhibited. This peptide carries four basic amino acid residues, perhaps explaining its weak ability to inhibit tPDE. Another peptide from the Pα subunit (38–49-Pα) only slightly inhibited tPDE. Peptide 37–49-Pβ had no influence on tPDE. These data support the notion that the effects of 24–46-Pγ and 46–87-Pγ on tPDE are specific.

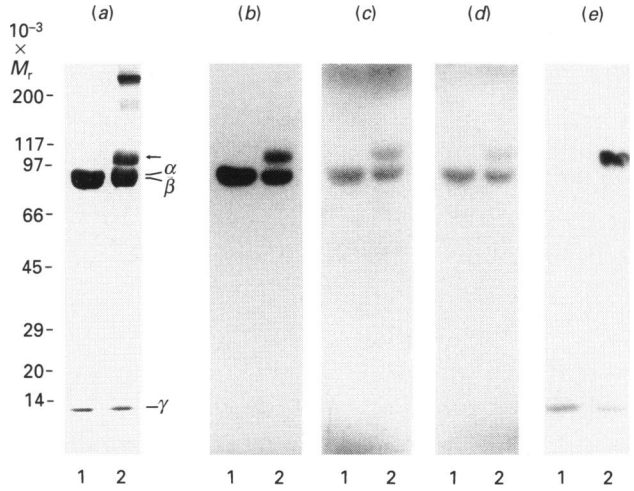

Fig. 9. Chemical cross-linking of Pγ to Pαβ with PDM

PDE was cross-linked with PDM, separated by SDS/10%-PAGE, transferred to nitrocellulose paper and analysed with anti-(PDE subunit)-specific antibodies as described in the Experimental section. PDE (1) or its cross-linked products (2) (*a*, Coomassie Blue-stained SDS/PAGE gel) were identified with anti-1–15-Pα (*b*), anti-(21–31 + 38–49)-Pα (*c*), anti-37–49-Pβ (*d*) anti-peptide antisera or anti-Pγ antisera (*e*).

Binding of 24-46-Pγ and 46–87-Pγ to Pαβ

It is known that Pγ binds to Pαβ with a very high affinity (K_i < 10 pM; Wensel & Stryer, 1986). To determine whether any of the Pγ fragments bind Pαβ, co-elution during h.p.l.c. gel filtration was used to study binding of ^{125}I-labelled Pγ fragments to PDE. This method allows the detection of only relatively strong binding (K_d in the nanomolar range). Both the central and *C*-terminal peptides bound Pαβ when this method was used. Fig. 8(*d*) shows that ^{125}I-labelled fusion protein almost entirely co-migrated with Pαβ, whereas no binding was observed after mixing with holo-PDE, which contains endogenous Pγ (Fig. 8*c*). Similar experiments demonstrated that both ^{125}I-Tyr-24–46-Pγ and 46–87-Pγ (^{125}I-Tyr^{84}) bound to Pαβ (Fig. 8*d*) with, however, a significantly lower affinity that Pγ. There was no binding of these polypeptides to holo-PDE (Fig. 8*c*), to trypsin–trypsin-inhibitor complex (Fig. 8*d*) or to BSA (results not shown). These data strongly suggest that 24–46-Pγ and 46–87-Pγ bind to specific sites on Pαβ that are blocked in holo-PDE by endogenous Pγ.

Cross-linking of Pγ to Pαβ

To obtain additional information on Pγ interaction with Pαβ, studies of cross-linking of Pγ to Pαβ were performed. PDM, a homobifunctional cross-linker of -SH groups, was chosen for these studies, since Pγ contains a single cysteine residue (Cys^{68}). A previous report demonstrated that PDM can cause Pγ to cross-link with Pβ, but no cross-linking product with Pα was found (Hingorani *et al.*, 1988). To address this question, we developed subunit-specific anti-PDE antibodies (Fig. 1).

Purified PDE was cross-linked with PDM and the composition of Pγ-containing products was analysed by Western immunoblotting using subunit-specific antisera. The cross-linking of PDE with PDM resulted in the formation of 100 kDa, 140 kDa, 190 kDa and higher-molecular-mass products. The cross-linking time and PDM concentration were optimized in

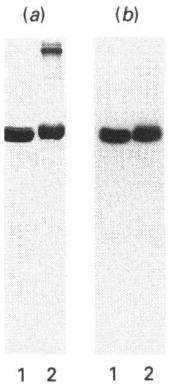

Fig. 10. Chemical cross-linking of tPDE with PDM

Lanes 1, tPDE; lanes 2, cross-linked tPDE. (*a*) Coomassie Blue-stained SDS/10% PAGE gel; (*b*) immunoblots with anti-1–15-Pα anti-peptide antisera.

preliminary experiments in order to obtain a better yield of the 100 kDa product, which includes Pγ (Fig. 9*a*). Under these conditions (15 min; 50 μM-PDM; 0.5 mg of PDE/ml) the 140 kDa product was not formed. Immunoblots with two anti-Pα, one anti-Pβ and anti-Pγ antibodies are shown in Figs. 9(*b*), 9(*c*) 9(*d*) and 9(*e*) respectively. The high-molecular-mass cross-linked products required a longer time for transfer to the nitrocellulose and therefore they are not present in the immunoblot.

All of the antibodies recognized the 100 kDa band, suggesting that the composition of this band is PαPγ/PβPγ. This result thus shows that Pγ binds not only to Pβ but also to Pα. To confirm that the 100 kDa band is composed only of cross-linked products of either catalytic subunit with Pγ and does not include intramolecularly linked or modified Pα and Pβ, tPDE was cross-linked with PDM. Limited proteolysis of PDE resulted in the absence of Pγ (Fig. 10*a*, line 1) and the 100 kDa cross-linked product is also missing (Figs. 10*a* and 10*b*, lanes 2). It also indicates that, after cleavage of the central polycationic domain, Cys^{68}-Pγ can no longer be cross-linked to P$\alpha\beta$. These data provide evidence that Cys^{68}-Pγ is involved in the cross-linking of Pγ to both Pα and Pβ catalytic subunits.

DISCUSSION

The Pγ-subunit of PDE plays a key role in photoinduced activation of the enzyme (Hurley & Stryer 1982). The availability of the Pγ sequence (Ovchinnikov *et al.*, 1986) has stimulated the study of its submolecular organization and its interaction with catalytic P$\alpha\beta$ subunits and α_t. We have studied the role of various Pγ regions in Pγ–P$\alpha\beta$ interaction. It appears that the Pγ *N*-terminal region does not participate in interaction with PDE catalytic subunits, since the 12–87-Pγ fragment was as effective as native Pγ and the fusion protein in tPDE inhibition.

There are several reasons to believe that the polycationic region, 24–46-*P*γ, is involved in binding to P$\alpha\beta$ and inhibiting enzyme activity. First, limited tryptic digestion of PDE leads to full enzyme activation (Hurley & Stryer, 1982). Trypsin cleaves Pγ at multiple sites within the polycationic site while leaving intact the large *C*-terminal fragment, 45–87-Pγ (Ovchinnikov *et al.*, 1986). Secondly, polycationic proteins are potent activators of PDE (Miki *et al.*, 1975) owing, perhaps, to their competition with the central region of Pγ for the binding site on P$\alpha\beta$.

In order to examine more directly the role of this polycationic site in Pγ function, we tested the effects of a synthetic peptide, 24–46-Pγ, on tPDE and basal PDE activity. We found that peptide 24–46-Pγ inhibited $\sim$ 50% of tPDE activity in a dose-dependent manner (K_i 15 μM). The peptide slightly stimulated basal enzyme activity. Previously it was shown that peptide 31–47-Pγ inhibited PDE activity at millimolar concentrations in solution (Morrison *et al.*, 1987). The difference in peptide potency in these two studies could be explained by the presence of intrinsic Pγ in the PDE sample. Alternatively, the region 24–33-Pγ was recently suggested to be essential for Pγ interaction with PDE catalytic subunits (Lipkin *et al.*, 1990*a*). The Pγ mutant Arg-24 $\rightarrow$ Gly was a weak inhibitor of tPDE activity, whereas mutations of Lys-41, -44 and -45 did not alter Pγ-inhibitory properties. These data imply that not only the polycationic nature of the 24–46-Pγ region, but also the location of basic amino acid residues within this region, are critical for Pγ function.

It is noteworthy that *C*-terminal truncated Pγ loses its ability to inhibit tPDE (Lipkin *et al.*, 1988; Brown & Stryer, 1989). How can this be reconciled with the finding that the 24–46-Pγ fragment, which is contained in the truncated Pγ, does inhibit tPDE? It might be explained by taking into account that the truncated Pγ was tested at similar concentrations as native Pγ, i.e. in the nanomolar range, whereas the concentration of 24–46-Pγ that produced inhibition was in the micromolar range.

The peptide not only inhibits PDE somewhat, but two types of evidence show that it binds tightly to P$\alpha\beta$. It can compete with Pγ for tPDE inhibition and it can also directly bind P$\alpha\beta$ and co-migrate on h.p.l.c. gel-filtration columns. This comigration of ^{125}I-Tyr-24–46-Pγ with tPDE over a column suggests a rather strong interaction, perhaps even stronger than could have been expected from the peptide inhibition of PDE activity ($K_i \sim 15$ μM). Such evidence of strong binding and the fact that 24–46-Pγ can not fully inhibit tPDE indicate that binding to P$\alpha\beta$ may be the primary role of this region.

The *C*-terminus of Pγ is also known to be important for Pγ function (Lipkin *et al.*, 1988; Brown & Stryer, 1989). A synthetic peptide corresponding to the sequence 73–87-Pγ inhibited tPDE activity at a concentration of $>$ 1 mM (Cunnick *et al.*, 1990). To gain further insight into the role of the Pγ *C*-terminus in the interaction with P$\alpha\beta$ we obtained a *C*-terminal fragment by exhaustive trypsin treatment of the fusion protein. This Pγ fragment was a very effective inhibitor of tPDE activity. The K_i of 46–87-Pγ (0.8 μM) was nearly 20-fold lower than that of 24–46-Pγ. This fragment could completely inhibit tPDE, compared with only $\sim$ 50% inhibition of tPDE by 24–46-Pγ. Also, 46–87-Pγ inhibited, while 24–46-Pγ weakly stimulated, basal PDE activity. These results suggest that the Pγ *C*-terminal region is a major contributor to P$\alpha\beta$ inhibition by Pγ. This fragment also was shown to bind to tPDE in h.p.l.c. gel-filtration experiments. The cross-linking studies reported here are also consistent with a role for the *C*-terminal region of Pγ in binding of P$\alpha\beta$. PDM, which cross-links cysteine residues in close proximity, cross-linked Pγ to both Pα and P$\alpha\beta$. Pγ has only one cysteine residue (Cys^{68}), located near the *C*-terminus. It is noteworthy that trypsin-treated Pγ could no longer be cross-linked to P$\alpha\beta$.

If two regions on Pγ are responsible for blocking PDE catalytic activity, what is the role of each site in this process? The following possibilities can be considered: (*a*) both sites participate in binding and inhibiting enzyme activity; (*b*) one site is more important for binding, whereas the other is necessary for inhibition; (*c*) only one site binds to P$\alpha\beta$; the second site does not directly interact with PDE but helps to maintain a proper conformation of the binding site.

Our data suggest that the regions 24–46 and 46–87 of Pγ possess both functions, binding and inhibition, and that Pγ has at least two sites of interaction with P$\alpha\beta$. However, 46–87-Pγ appears to be the most important for Pγ's inhibitory function.

Quite possibly a significant part of Pγ is in close proximity with Pαβ. Such an organization of Pγ–Pαβ interaction would provide an explanation for the high affinity of intersubunit binding. It would also help to explain why tryptic cleavage and *C*-terminal truncation of Pγ both lead to PDE activation. Trypsin cleaves Pγ within one of the major binding sites and causes dissociation of the *C*-terminal half of the molecule, which then has a lower affinity for Pαβ than Pγ. *C*-terminal truncation of Pγ removes (partially or completely) a region important for both binding to Pαβ and inhibition of PDE activity.

A detailed study of the Pγ interaction with Pαβ is relevant for understanding the mechanism of G_t activation of PDE. It is known that binding of G_t to PDE can accelerate the dissociation of Pγ from inhibitory sites (Deterre *et al.*, 1988; Wensel & Stryer, 1990), i.e. decrease its affinity for these sites. The polycationic region 24–46-Pγ that we have found to partipicate in binding to Pαβ has previously been implicated as a site of Pγ-α_tGTP interaction. Monoclonal antibodies which blocked the 24–45-Pγ site prevented the photoactivation of PDE (Lipkin *et al.*, 1988). A synthetic peptide corresponding to amino acids 31–45-Pγ inhibited the GTPase activity of α_t ($IC_{50} \sim 450\ \mu M$) by affecting its binding to the photoreceptor membrane and by altering GDP–GTP exchange (Morrison *et al.*, 1987, 1989). Binding of α_t near one region of Pγ–Pαβ interaction, followed by a conformational change of the Pγ *C*-terminus, are likely to be the steps leading to Pγ dissociation or displacement from inhibitory sites on PDE.

If α_t and Pαβ bind within one region on Pγ, they would most likely be close to each other during the enzyme-activation process. Such proximity of α_t and Pαβ is consistent with results of Kroll *et al.* (1989) and the formation of the cross-linking products between these proteins (Hingorani *et al.*, 1988). However, whether α_tGTP interacts with Pαβ in addition to its interaction with Pγ is not yet clear.

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