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Subunit Stoichiometry of Retinal Rod cGMP Phosphodiesterase†

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ABSTRACT: The cyclic GMP phosphodiesterase of the retinal rod is composed of three distinct types of polypeptides: α (90 kDa), β (86 kDa), and γ (10 kDa). The γ subunit has been shown to inhibit phosphodiesterase activity associated with α and β. To investigate the subunit stoichiometry of the retinal phosphodiesterase, we have developed a panel of monoclonal and peptide antibodies that recognize individual phosphodiesterase subunits. By quantitative and immunochemical analysis of the purified subunits, we have shown that each phosphodiesterase molecule contains one copy each of α and β subunit and two copies of γ subunit. Moreover, γ can be chemically cross-linked to both α and β, but not to itself, suggesting that α and β may each bind one γ. The phosphodiesterase is fully activated when both copies of γ were removed by proteolysis with trypsin. Upon recombination of the purified γ subunit with the trypsin-activated phosphodiesterase containing $\alpha\beta$, the $\alpha\beta\gamma_2$ stoichiometry is once again restored, with concomitant total inhibition of activity. Our results suggest that at least two activated transducin molecules are required to fully activate one molecule of phosphodiesterase in retinal rods.

Retinal phosphodiesterase (PDE),[1] which serves to regulate the cytosolic cGMP concentration of vertebrate retinal rods, is one of several key proteins involved in visual excitation (Chabre, 1985; Fung, 1986; Stryer, 1986; Hurley, 1987; Liebman et al., 1987). It is a peripheral membrane protein consisting of α (90 kDa), β (86 kDa), and γ (10 kDa) polypeptides (Baehr et al., 1979). In this multimeric form, the hydrolytic activity associated with $\alpha\beta$ is inhibited by γ (Hurley & Stryer, 1982). The inhibition is relieved when the γ subunit is removed by the GTP-bound form of the retinal G-protein transducin (Fung et al., 1981; Wensel & Stryer, 1986; Deterre et al., 1986; Fung & Griswold-Prenner, 1989), leading to a transient reduction of the intracellular level of cGMP in ROS (Miki et al., 1975; Yee & Liebman, 1978; Woodruff & Bownds, 1979; Blazynski & Cohen, 1986; Cote et al., 1986) and the closure of many cGMP-sensitive cation channels at the plasma membranes (Fesenko et al., 1985; Yau & Nakatani, 1985). As a result, the influx of Na^+ through the plasma membrane decreases (Hagins et al., 1970; Baylor et al., 1979), and the rod hyperpolarizes (Tomita, 1970).

Although rod PDE has been purified to homogeneity (Miki et al., 1975; Baehr et al., 1979) and the amino acid sequences of both α- and γ-polypeptides have been deduced from their corresponding cDNA (Ovchinnikov et al., 1986, 1987), the relative stoichiometry of α, β, and γ is still not well-defined. PDE has always been assumed to be composed of α- and β-polypeptides containing one to two copies of γ. Evidence supporting this stoichiometry comes from the observations that the molecular weight of PDE determined by gel filtration or by sucrose density centrifugation is approximately 170000 and that the Coomassie Blue staining patterns of the PDE α and β chains separated by SDS-PAGE are consistently equally intense (Baehr et al., 1979). These findings, however, do not provide information on the number of copies of γ per PDE molecule, nor do they exclude the possibility that purified PDE is a mixture consisting of equal amounts of isozyme containing either α or β. In agreement with this latter possible interpretation, Hurwitz et al. (1985) have reported the chromatographic separation of the β subunit on a Mono-Q anion-exchange column.

In this paper, we demonstrate by immunoprecipitation of the PDE holoenzyme with α-specific monoclonal antibodies and by quantitative Western blot analyses that the stoichiometry of purified rod PDE is $\alpha\beta\gamma_2$. We further support this finding by showing that total inhibition of the hydrolytic activity of $\alpha\beta$ requires two γ per $\alpha\beta$. Finally, we study the topological organization of the α-, β-, and γ-polypeptides in a qualitative manner by specifically cross-linking the PDE subunits using the homobifunctional cross-linker dimethyl suberimidate, which results in the successive formation of a protein product containing first one and then two copies of γ cross-linked to each of the α and β subunits. While this work was in progress, Deterre et al. (1988) have reported the chromatographic separation of two populations of activated PDE, one with about 50% of the γ content of native PDE and the other totally devoid of γ. Whalen and Bitensky (1989) have also demonstrated two classes of γ binding sites per $\alpha\beta$

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[1] Abbreviations: GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); MOPS, morpholinopropanesulfonic acid; mAb, monoclonal antibody; PDE, retinal cGMP phosphodiesterase; ROS, rod outer segment(s); TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; T_α, the α subunit of transducin; α, β, and γ, subunits of phosphodiesterase.

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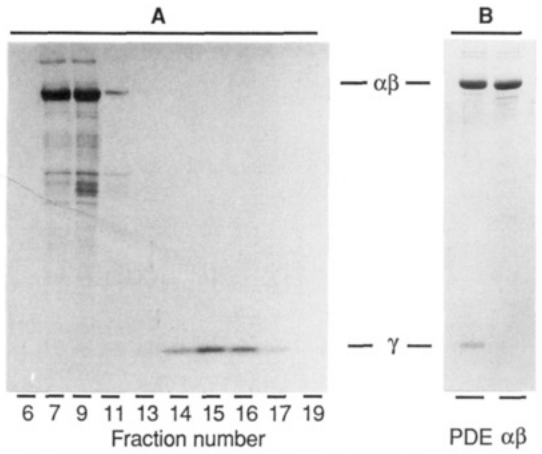

FIGURE 1: SDS–PAGE analysis of purified PDE subunits. (Panel A) Analysis of 7 μL of each fraction obtained by gel filtration on a Bio-Gel P-100 column of partially purified PDE in 0.2 M formic acid. The peak fractions containing the γ subunit (15 and 16) were pooled and used in the experiments. (Panel B) Analysis of 6 μg of purified PDE and trypsin-activated PDE. Proteins were visualized by Coomassie Blue staining.

in both the frog and bovine rod PDE. These important findings, together with the quantitative analysis of the subunit stoichiometry reported here, provide the strongest evidence to date that rod PDE contains two copies of the inhibitory γ subunit.

Experimental Procedures

Materials. Frozen bovine retinas were purchased from J. A. Lawson Co., Lincoln, NE. Protein A and cyanogen bromide activated Sepharose were obtained from Pharmacia. Pansorbin was a product of Calbiochem. TPCK-trypsin was purchased from Worthington. Immobilized TPCK-trypsin and dimethyl suberimidate were from Pierce. Biotinylated second antibody and avidin–peroxidase were products of Vector Laboratories. ^{125}I-Labeled protein A was prepared by iodination with chloramine T. Monoclonal antibodies immobilized on cyanogen bromide activated Sepharose were prepared as described by the manufacturer. ROS membranes were isolated as described previously (Fung, 1983).

The compositions of the buffered solutions were as follows: buffer A, 10 mM MOPS, pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, and 200 mM NaCl; buffer B, 10 mM *N*-(2-hydroxyethyl)-piperazinepropanesulfonic acid, pH 8.0, 0.2 M NaCl, and 1 mM DTT; buffer C, 20 mM Tris, pH 7.5, 0.5 M NaCl, and 0.05% Tween 20.

Preparation of Phosphodiesterase Subunits. Retinal PDE was extracted from photolyzed ROS membranes and purified by column chromatography as described previously (Baehr et al., 1979; Fung & Nash, 1983). PDE obtained by these procedures comprised approximately 97–98% of the total proteins, as determined by densitometric scanning of the proteins separated by SDS–PAGE and stained with Coomassie Blue (Figure 1B, lane 1).

The inhibitory γ subunit was isolated according to a modified procedure of Hurley and Stryer (1982). Briefly, 15 mg of partially purified PDE obtained by DEAE-Sephadex column chromatography (Baehr et al., 1979) was concentrated by ultrafiltration (Amicon type YM-100) to 2 mL. This step was found to remove proteins of mass less than 30 kDa from the PDE extract. The protein concentrate was then diluted to 2 mg/mL with buffer A and added dropwise to an equal volume of 0.2 M formic acid, pH 2.3. The mixture was heated at 70 °C for 15 min, allowed to cool to room temperature, and centrifuged at 13000*g* for 20 min. The supernatant, which contained approximately 85% PDE, was then concentrated to about 25 mg/mL using a Centricon 10 microconcentrator (Amicon), loaded onto a Bio-Gel P-100 column (0.6 × 75 cm), and eluted with 0.2 M formic acid at a flow rate of 2 mL/h. Fractions (0.4 mL) were collected and analyzed by SDS–PAGE. As shown in Figure 1A, the inhibitory γ subunit was clearly separated from αβ and was judged to be at least 95–98% pure. The peak fractions containing γ were pooled and stored in formic acid at 4 °C. γ remained active for several weeks under this condition.

To prepare trypsin-activated PDE containing only the αβ-polypeptides, purified PDE (1 mg/mL) in buffer A was allowed to pass slowly through a small column (0.15 mL) of immobilized TPCK-trypsin (Hurley & Stryer, 1982). Fractions of 0.2 mL were collected, and those containing phosphodiesterase activity were pooled and stored frozen at −20 °C. By adjusting the flow rate at which the PDE passed through the column of the immobilized trypsin, γ was destroyed and removed (Figure 1B, lane 2). The total removal of γ was further confirmed by Western blot analysis of the cleaved PDE using an anti-γ antibody (Figure 6, lane 2) which is capable of detecting less than 10 ng of γ. Although trypsin is known to degrade the membrane binding domain of PDE (Wensel & Stryer, 1986; Whalen & Bitensky, 1989) by removing a small region from the carboxyl terminus of the α subunit (Ong et al., 1989), it does not appear to affect the γ binding sites of the αβ subunits. The trypsin-activated PDE (Figure 1B, lane 2) exhibited approximately 95% of maximal phosphodiesterase activity and was capable of interacting with purified γ (as described later).

Production of Monoclonal Antibodies. The preparation of monoclonal antibodies directed against PDE was essentially the same as previously described (Navon & Fung, 1988). Briefly, CAF1 mice were each immunized with a total of 0.4 mg of purified PDE according to the method of Stähli et al. (1980). Fusion, plating, and subcloning were carried out as described by Harwell et al. (1984) with RPMI 1640 containing glutamine as the medium for cell culture. CAF1 mice were the source of peritoneal feeder cells. To screen antibody-producing hybridomas, 50 μL of supernatant from each culture well was transferred to polyvinyl 96-well assay plates coated with PDE. After 1 h at 23 °C, bound monoclonal antibodies were detected by ELISA with biotinylated second antibody and avidin–peroxidase. Large amounts of monoclonal antibodies were obtained from the ascites fluid of CAF1 mice according to the protocol of Brodeur et al. (1984). All monoclonal antibodies were purified from the ascites fluid by chromatography on either DEAE-Affi Blue (Bruck et al., 1982) or protein A conjugated agarose. The chain class of each monoclonal antibody was determined by an immunoperoxidase sandwich assay in a commercially available kit (American Qualex).

Preparation of Antipeptide Antibodies. Peptides with sequences corresponding to 24–41 and 44–54 of γ (Ovchinnikov et al., 1986) were synthesized by the Merrifield solid-phase method at the Peptide Synthesis Facility at UCLA. Their sequences were as follows: γ_{24-41}, Arg-Lys-Gly-Pro-Pro-Lys-Phe-Lys-Gln-Arg-Gln-Thr-Arg-Gln-Phe-Lys-Ser-Lys; γ_{44-54Y}, Lys-Lys-Gly-Val-Gln-Gly-Phe-Gly-Asp-Asp-Ile-Tyr. The latter was synthesized with a carboxyl-terminal tyrosine as a potential site for radioiodination. Each peptide was purified by gel filtration on a Bio-Gel P2 column, diluted to 2.5 mg/mL with 0.2 M sodium phosphate, pH 7.5, and conjugated to keyhole limpet hemocyanin (2.5 mg/mL) with 0.05% glutaraldehyde. After 1 h of incubation at room temperature and

overnight at 4 °C, the coupling reaction was terminated by the addition of an excess amount of ethanolamine. Without further purification, the resulting conjugate was used to immunize rabbits. Briefly, conjugated peptide (0.5 mg/rabbit) in complete Freund's adjuvant was injected subcutaneously at multiple points into New Zealand White rabbits. The injection was repeated every 4 weeks. Ten days after the second injection, the rabbits were bled and the sera tested for antipeptide antibodies by solid-phase radioimmune assay using peptide-coated microtiter plates. The subunit specificity of the antisera was analyzed on Western blots. Antipeptide antibodies were then purified from positive sera by affinity chromatography on peptide conjugated to cyanogen bromide activated Sepharose.

Calibration of Subunit Concentrations. To generate a calibration curve for quantitative Western blot analysis, the concentration of purified γ in a standard preparation was accurately determined by two methods. First, four samples of γ of known volume were subjected to quantitative amino acid analysis. Thirteen different sets of γ concentrations were obtained by dividing the amount of each amino acid determined with the known number of residues in γ (Ovchinnikov et al., 1986), and an average molar concentration was calculated. Second, the amount of cysteine in four γ samples of known volume was determined. Since γ contains only one cysteine residue, this analysis provides an independent and accurate measurement of the molar concentration of γ. Essentially the same concentration of γ was obtained by both methods in three independent analyses. The variations in concentration determination were less than 10% between the two methods and less than 15% between different analyses.

Gravimetric analysis was used to determine accurately the concentration of $\alpha\beta$. In a typical experiment, several milligrams of either purified PDE or $\alpha\beta$ were exhaustively dialyzed against 0.2 M formic acid, lyophilized, and weighed. The sample was then dissolved in a known volume of 0.2 M formic acid and used as a standard. To ensure the accuracy of this procedure, the concentration of $\alpha\beta$ in this standard sample was independently established by quantitative amino acid analysis and from empirical calculations using its absorbances at 210 nm (Tombs et al., 1959) and 215/225 nm (Wolf & Maguire, 1983). All three procedures gave the same concentration for PDE, with variations less than 10%.

Quantitative Western Blot Analysis. To quantitate the amount of each subunit, a sample of purified PDE was subjected to Western blot analysis and detected with ^{125}I-protein A (Towbin et al., 1979; Navon et al., 1986). The amount of protein present in each band was determined by comparing its radioactivity with a standard curve generated by using known amounts of PDE subunits processed on the same blot. Alternatively, an autoradiogram of the Western blot was developed and scanned by a densitometer. The integrated area associated with each PDE subunit band was determined and compared to those associated with known amounts of the PDE standards of the calibration curve. Both procedures gave the same results.

Determination of the Subunit Stoichiometry of Reconstituted PDE. Reconstituted PDE was prepared by titrating 30 μL of 1 mg/mL trypsin-activated PDE in buffer A with NaOH-neutralized γ until the phosphodiesterase activity was completely inhibited. After 30 min of incubation at 21 °C, 0.2 mL of immobilized mAb PDE812 containing 4 mg/mL bovine serum albumin in buffer A was added, and the mixture was incubated for one additional hour. The PDE–mAb complex was pelleted by centrifugation (5 min, 5000g) and washed twice with buffer C. Bound PDE was eluted from the immobilized mAb PDE812 by resuspending the pellets in 150 μL of gel electrophoresis sample buffer (Laemmli, 1970) and heated to 70 °C for 10 min. The suspension was cleared by centrifugation, and the supernatant was immediately analyzed by SDS–PAGE and Western blot.

Chemical Cross-Linking with Dimethyl Suberimidate. PDE subunits were cross-linked with the homobifunctional cross-linker dimethyl suberimidate under the following conditions: purified PDE was exchanged into buffer B by dialysis, adjusted to a concentration of 2 mg/mL, and added to an equal volume of dimethyl suberimidate dissolved in the same buffer immediately prior to the cross-linking reaction. The reaction was allowed to proceed for varying periods at room temperature and was quenched by addition of a 100-fold excess of ammonium chloride. The cross-linked products were analyzed by SDS–PAGE and immunoblotted with subunit-specific antibodies to identify the cross-linked subunits. Control experiments substituting the one-sided imidoester ethyl acetimidate for dimethyl suberimidate, in one case, and trypsin-activated PDE for the PDE holoenzyme, in another, were performed to verify that the change in electrophoretic mobility was not artifactual.

Assay for Phosphodiesterase Activity. Phosphodiesterase activity was determined by measuring proton release due to cyclic GMP hydrolysis (Yee & Liebman, 1978). A sample (120 μL) of activated PDE in buffer A was placed in the well of the microtiter plate, and the reaction was initiated by injecting 80 μL of 5 mM cGMP. The change in pH was monitored with a pH microelectrode (Microelectrodes, Inc., Londonderry, NH) and then displayed on a strip chart recorder.

Assay for Inhibitory Activity. The inhibitory activity of γ was routinely assayed by the following procedure. An aliquot (usually 1 μL) of purified γ in 0.2 M formic acid was first placed in the well of a microtiter plate. An equal volume of 0.2 M NaOH was then carefully added to a separate area of the same well. The two samples were then rapidly mixed by adding 78 μL of buffer A, followed immediately by 40 μL of 0.017 mg/mL trypsin-activated phosphodiesterase. After 3 min of incubation, an aliquot of 80 μL of 5 mM cGMP was added, and the residual phosphodiesterase activity was then measured.

Analytical Methods. Protein concentrations were routinely determined by the method of Coomassie Blue binding (Bradford, 1976) with γ-globulin from Bio-Rad Laboratories as the standard. SDS–polyacrylamide gel (13%) electrophoresis was performed by the method of Laemmli (1970). Lysozyme (14 400), soybean trypsin inhibitor (21 500), carbonic anhydrase (31 000), ovalbumin (45 000), bovine serum albumin (68 000), and phosphorylase B (92 500) were used as molecular weight standards. Amino acid analysis was performed by the Protein Sequencing Facility at UCLA.

Results

α, β, and γ Chains of PDE Are Distinguishable by Monoclonal and Antipeptide Antibodies. A panel of monoclonal antibodies was developed to assist in the quantitation and identification of individual subunits of PDE. During the course of 2 separate fusions using purified bovine PDE as an antigen, over 10 antibody-producing hybridomas were identified. Of these, five distinct PDE-positive cell lines were characterized and injected into mice to produce antibody-containing ascitic fluids. Their specificities and subtypes are summarized in Table I. The characterization of these monoclonal antibodies and their effects on phosphodiesterase activity will be published

Table I: Monoclonal Antibodies against the Subunits of PDE[a]

clone	isotype	specificity: subunit	specificity: conformation
PDE922	IgG_1	β	native/denatured
PDE1331	IgM	α	denatured
PDE812	IgG_{2b}	α	native/denatured
PDE1852	IgG_1	α	native/denatured
PDE3413	IgG_1	α and β	denatured

[a] Assays for chain and binding specificity are described under Experimental Procedures. The specificity of the monoclonal antibodies was determined by solid-phase radioimmune assay, Western blotting, and immunoprecipitation (Navon & Fung, 1988).

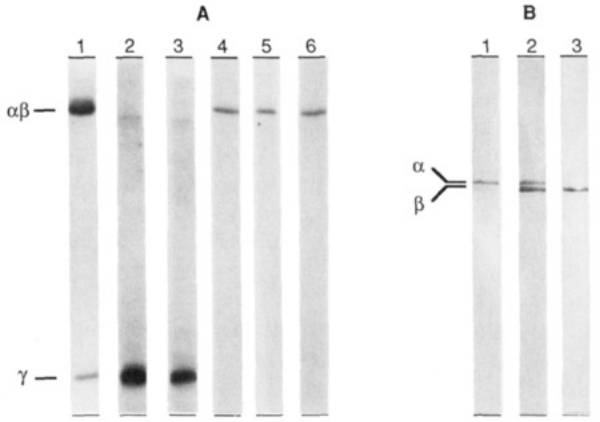

FIGURE 2: Antibody specificities. (Panel A) Western blot of PDE using rabbit anti-PDE antiserum (lane 1), affinity-purified anti-γ_{24-41} antibodies (lane 2), affinity-purified anti-γ_{44-54Y} antibodies (lane 3), mAb PDE1331 (lane 4), mAb PDE812 (lane 5), and mAb PDE922 (lane 6) and detected with ^{125}I protein A. (Panel B) Western blot of resolved α- and β-polypeptides of PDE analyzed with mAb PDE1331 (lane 1), a mixture of mAb PDE922 and mAb PDE1331 (lane 2), and mAb PDE922 (lane 3). The α and β subunits were separated by prolonged electrophoresis on a 13% SDS–polyacrylamide gel and detected by the Vectastain ABC method.

elsewhere. In the present study, three subunit-specific monoclonal antibodies, PDE1331, PDE812, and PDE922, were used either as probes or as affinity matrices to characterize the subunit composition of PDE (as will be described later). As shown on Western blot (Figure 2A), polyclonal anti-PDE antibodies (lane 1) produced in rabbits detected all three subunits of bovine PDE. In contrast, mAb PDE1331 (lane 4), mAb PDE812 (lane 5), and mAb PDE922 (lane 6) reacted only with the large $\alpha\beta$ catalytic subunits, and not the inhibitory γ subunit. Their subunit specificities were further defined on Western blots in which α and β were resolved, and then detected with biotinylated antibody and avidin-conjugated horseradish peroxidase (Vectastain ABC method) to increase resolution. As shown in Figure 2B, mAb PDE1331 (lane 1) recognized an immunoreactive protein band that migrates slightly slower than the protein band recognized by mAb PDE922 (lane 3). When the same blot was tested with a mixture containing both PDE922 and PDE1331 (lane 2), two immunoreactive bands corresponding to the α and β chains were detected. This result confirmed that mAb PDE1331 recognizes the α chain, whereas mAb PDE922 is specific for β. The same procedure was used to identify the subunit specificity of all the PDE-positive monoclonal antibodies (Table I).

Despite the use of heteromeric PDE to immunize mice, which were subsequently found to produce positive immune sera against all three polypeptide chains of PDE, none of the PDE-positive hybridomas were found to produce antibodies specifically against the inhibitory γ subunit. To obtain antibodies directed against γ, rabbit antisera against synthetic

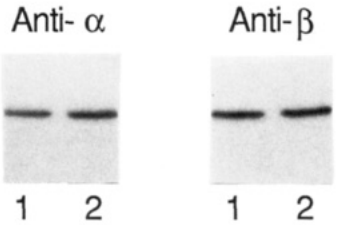

FIGURE 3: Relative amount of α and β subunits before and after immunoprecipitation. Purified PDE was immunoprecipitated with α-specific mAb PDE812 immobilized on cyanogen bromide activated Sepharose, washed extensively with buffer A containing 1% NP-40, and then eluted with 1% SDS. The subunit content of PDE before immunoprecipitation (lane 1) and the SDS eluent (lane 2) were analyzed on Western blot with β-specific mAb PDE922 (left panel) and α-specific mAb PDE1331 (right panel).

peptides corresponding to amino acid residues 24–41 (γ_{24-41}) and 44–54 (γ_{44-54Y}) were developed. Both antisera showed positive reaction in radioimmune assays performed in polyvinyl wells coated with unconjugated peptides (data not shown). Moreover, both types of antipeptide antibodies cross-reacted with γ on Western blots and showed little immunochemical reactivity toward α and β (Figure 2A, lanes 2 and 3).

PDE Is Composed of α, β, and γ. PDE isolated from bovine ROS is thought to be composed of both α and β chains, based mainly on Coomassie Blue staining intensity of the PDE subunits separated on SDS–polyacrylamide gels. However, this type of analysis does not rule out the possibility that purified PDE in fact consists of two populations of closely related isozymes, each containing either the α or the β chains. In order to distinguish these two alternative possibilities, purified PDE was allowed to bind to immobilized mAb PDE812 that recognized only the α chain (Table I), washed exhaustively with buffer A containing 1% NP-40, and then eluted with a 1% SDS solution. If PDE is composed of a mixture of isozymes, this procedure would have selectively depleted the β chain. Analyses of the PDE eluents with β-specific mAb PDE922 and α-specific mAb PDE1331 (Figure 3), however, indicated that the relative amounts of α and β chain before and after the affinity purification were identical. This result excluded the possibility that the purified PDE is a mixture and demonstrated that PDE is composed of one copy each of α and β.

Each PDE Contains Two Copies of γ. Quantitative Western blot analyses using ^{125}I protein A for detection were employed to determine the subunit stoichiometry of PDE. The amount of each subunit present in purified PDE was determined by comparing its radioactivity with a standard curve generated by PDE subunit standards processed on the same Western blot. As shown by the γ calibration curve (Figure 4A), the radioactivity associated with the γ band was linear from 0 to 40 pmol. When a known amount of purified PDE (Figure 4B) as a standard and rabbit anti-PDE antibodies for detection were used, a similar calibration curve for $\alpha\beta$ in the range from 0 to 15 pmol was also established. From the two standard curves, the amount of γ and $\alpha\beta$ associated with an identical sample of purified PDE processed at the same time as the calibration standards (Figure 4, right panel) was respectively 16.5 and 8.5 pmol, which gave a α:β:γ molar ratio of 1:1:1.95. These results indicate that there are 2 mol of γ per mole of α and β.

Binding of Two γ Completely Inhibits the Activity of Trypsin-Activated PDE. As shown previously (Hurley & Stryer, 1982), PDE can be maximally activated by proteolysis with immobilized trypsin. As judged by SDS–PAGE, the activated PDE consists of $\alpha\beta$ and a minute amount of a 70-

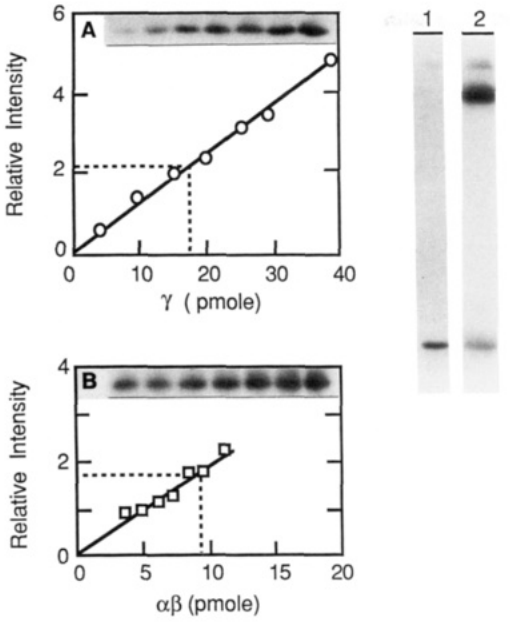

FIGURE 4: Quantitative Western blot analysis of subunit stoichiometry. Increasing amounts of purified γ (panel A) were subjected to Western blot analysis and detected with affinity-purified anti-γ_{24-41} antibodies as described under Experimental Procedures. The calibration curve was constructed from the radioactivity associated with the γ band measured with an AMBIS radioactivity scanner (inset of panel A). The dashed line in panel A represents the relative amount of radioactivity associated with the γ band of PDE (lane 1) processed at the same time as the calibration standards. The calibration curve for αβ (panel B) was determined by the same procedure and detected with rabbit anti-PDE antisera. The dashed line in panel B represents the relative radioactivity associated with the αβ bands of PDE (lane 2). The amounts of PDE in lane 1 and lane 2 are identical.

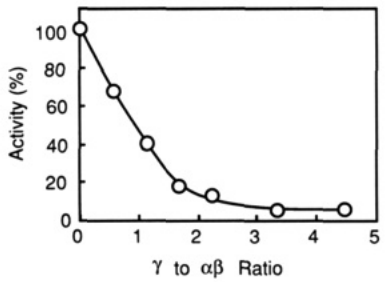

FIGURE 5: Inhibition of trypsin-activated PDE with purified γ. The activities of trypsin-activated PDE (0.7 μg) were measured in the presence of increasing amounts of γ as described under Experimental Procedures and are plotted as a function of the molar ratio of γ to αβ.

kDa fragment (Hurley & Stryer, 1982) but is totally devoid of γ (Figure 1B, lane 2). Upon the recombination of γ, the phosphodiesterase activity is again inhibited. To determine how many copies of γ are required for total inhibition, the phosphodiesterase activity of trypsin-treated PDE was measured as a function of γ added. As shown in Figure 5, the activity decreases with increasing amounts of γ added. A 90% inhibition of the activated phosphodiesterase activity was achieved with 1.9 mol of γ per mole of activated PDE.

To further ascertain the number of bound γ, the reconstituted PDE was separated from the excess γ by immunoprecipitation with immobilized mAb PDE812, and the amounts of γ and αβ in the immunoprecipitate were quantified on Western blot by using a mixture of anti-PDE and anti-γ antibodies to enhance the detection of the γ subunit. To simplify the quantitation, the radioactivities associated with the γ and αβ bands were compared with the native uncleaved PDE, which was earlier shown to contain two γ. As shown in Figure

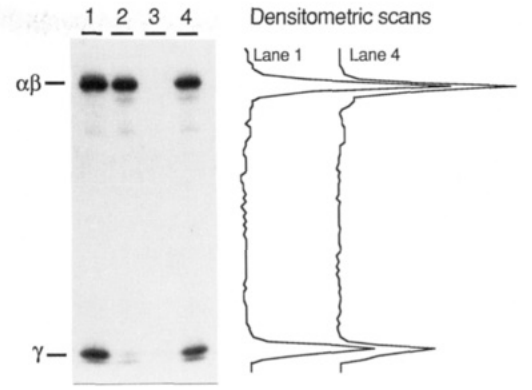

FIGURE 6: Subunit stoichiometry of reconstituted PDE. (Panel A) Native PDE (lane 1), trypsin-activated PDE (lane 2), purified γ (lane 3), and reconstituted PDE (lane 4) were immunoprecipitated with mAb PDE812 attached to Pansorbin, washed extensively with buffer C, and then eluted with 1% SDS. The subunit content of the immunoprecipitates was visualized on Western blots using a mixture of anti-γ_{24-41} and rabbit anti-PDE antibodies. (Panel B) Densitometric scans of lanes 1 and 4 showing nearly equal ratios of γ to αβ in native and reconstituted PDE.

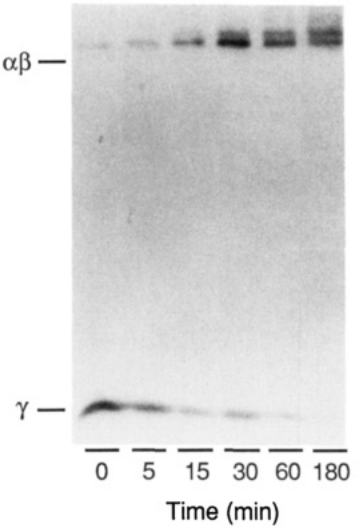

FIGURE 7: Time course of γ cross-linking to αβ with dimethyl suberimidate. PDE subunits were cross-linked with dimethyl suberimidate as described under Experimental Procedures. The products were analyzed on Western blot detected with affinity-purified anti-γ_{24-41} antibodies.

6, native PDE (lane 1), trypsin-activated PDE (lane 2), and reconstituted PDE (lane 4) were quantitatively precipitated by immobilized mAb PDE812. In contrast, free γ (lane 3) was not precipitated. Hence, the γ subunit which coprecipitated with the reconstituted PDE (lane 4) must be tightly associated with αβ. Moreover, the relative amounts of αβ to γ in both native and reconstituted PDE immunoprecipitates, as determined by densitometric scanning of the autoradiogram, were essentially identical (Figure 6B). These analyses confirm that upon the inhibition of the phosphodiesterase activity each PDE molecule contained two copies of γ.

Dimethyl Suberimidate Cross-Links γ to both α and β. Nearest-neighbor analysis by chemical cross-linking was employed to gain further insight into the subunit configuration of PDE. Several cross-linkers were tested, including a number of diimidate cross-linkers of various lengths and glutaraldehyde, and in each case the composition of the cross-linked products was identified on Western blots by using subunit-specific antibodies. Figure 7 shows the results of a typical experiment using dimethyl suberimidate for cross-linking and anti-γ antibodies for detection. The time course of cross-

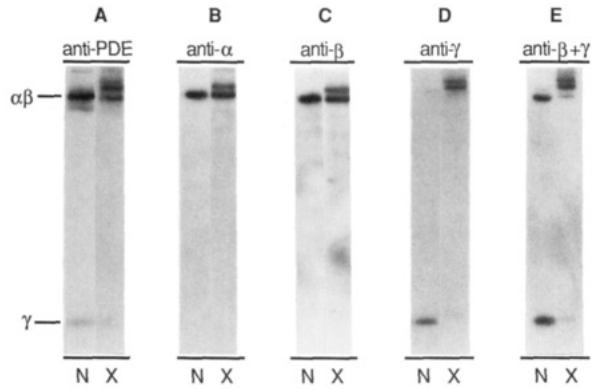

FIGURE 8: Identification of the cross-linked subunits. PDE subunits were cross-linked with dimethyl suberimidate and analyzed on Western blots as described under Experimental Procedures. Native PDE (N) and its cross-linked products (X) were then detected with rabbit anti-PDE antiserum (panel A), mAb PDE1331 (panel B), mAb PDE922 (panel C), anti-γ_{24-41} antibodies (panel D), and a mixture of anti-γ_{24-41} antibodies and mAb PDE 922 (panel E).

linking PDE with dimethyl suberimidate reveals the disappearance of the γ band and the concomitant formation of a 100-kDa γ-containing cross-linked product migrating slightly slower than $\alpha\beta$. Prolonged cross-linking led to the formation of an additional high molecular weight protein band differing from the first one by approximately 10 kDa, and finally to very high molecular mass aggregates. The cross-linking of γ with dimethyl suberimidate was highly specific. High molecular weight cross-linked protein bands were not observed in control experiments substituting the one-sided imidoester ethyl acetimidate for dimethyl suberimidate in one case, and trypsin-activated PDE for the PDE holoenzyme in another. Moreover, the γ dimer with predicted molecular weight of 26K was never detected. This result suggests that the two γ subunits were cross-linked to the catalytic $\alpha\beta$ subunit, and not to each other.

To further identify the subunit composition of the cross-linked products, PDE was incubated with dimethyl suberimidate for 1 h, and the reaction products were then analyzed on Western blots with either rabbit anti-PDE antibodies (Figure 8, panel A) or subunit-specific antibodies (Figure 8, panels B–D). As shown in panel A, in addition to $\alpha\beta$, a major protein band with apparent molecular weight of 100K was detected with anti-PDE antibodies. Moreover, a faint 110-kDa band trailing slightly behind the major cross-linked protein product was also apparent. When the same mixture was analyzed with subunit-specific antibodies, the 100-kDa protein band was found to be immunoreactive to mAb PDE922 (panel B), mAb PDE1331 (panel C), and anti-γ_{24-41} antibodies (panel D). This high molecular weight cross-linked product was also distinct from native β, as shown on Western blot by using a mixture of mAb PDE922 and anti-γ_{24-41} antibodies (panel E). These results indicate that the 100-kDa protein band is derived from cross-linking γ to either α or β.

In addition to the 100-kDa cross-linked product, the γ-specific antipeptide antibodies, but not the α- or β-specific monoclonal antibodies, detected a minor immunoreactive band of apparent molecular weight 110K (Figure 7 and Figure 8, panel D). On the basis of its molecular weight and assuming that the additional cross-linking may have simply masked the epitopes of the monoclonal antibodies, this protein band is most likely formed by cross-linking two γ to either α or β.

Discussion

PDE in the dark-adapted rod outer segment is inhibited, but can be activated by light in the presence of GTP (Wheeler et al., 1977; Yee & Liebman, 1978; Fung et al., 1981) or, alternatively, by tryptic proteolysis which selectively removes the γ subunit (Hurley & Stryer, 1982). These findings, together with the observed inhibitory effect of γ, have led to the initial proposal that transducin activates PDE by relieving an inhibitory constraint exerted by γ (Hurley & Stryer, 1982; Yamazaki et al., 1983; Deterre et al., 1986). Recently, we (Fung & Griswold-Prenner, 1989) have demonstrated that γ binds to the T_α subunit of transducin in a nucleotide-dependent manner, thus providing definitive evidence for a central role for γ in the light regulation of phosphodiesterase activity. In this study, we have demonstrated that bovine rod PDE contains two copies of γ and eliminated the possibility that isolated PDE is a mixture consisting of an equal amount of $\alpha_2\gamma_2$ and $\beta_2\gamma_2$ isozymes. Our results are in excellent agreement with those of Deterre et al. (1988) and Whalen and Bitensky (1989), who have recently reached the same conclusion using completely different approaches.

Our strategy was to measure independently the amount of γ and $\alpha\beta$ in any given PDE preparation using quantitative Western blot analysis. To generate a calibration curve relating the amount of γ to the measured radioactivity on Western blot, we determined accurately by quantitative amino acid analysis the molar concentration of purified γ in a standard solution. This approach was facilitated by the fact that the amino acid sequence of γ is known (Ovchinnikov et al., 1986). Since the amino acid composition of the β chain is still not yet available, we generated a calibration curve for $\alpha\beta$ from a standard solution determined by precise gravimetric analysis. With the use of standard solutions of PDE calibrated by these methods, the α:β:γ molar ratio of several different PDE preparations was estimated to be 1:1:1.95. Although gravimetric analysis was deemed the most accurate, the large amount of purified PDE required for this determination precluded its continuous repetition. Consequently, the molar concentration of an $\alpha\beta$ standard solution was independently verified by total amino acid analyses and from the absorbance in the 200–220-nm region (as described under Experimental Procedures). The results of these measurements were found to be within 10% of that measured gravimetrically. For example, calculations of the subunit stoichiometry based on the absorbances at 210 nm (Tombs et al., 1959) and 215/225 nm (Wolf & Maguire, 1983) gave a α:β:γ molar ratio of 1:1:1.97 and 1:1:1.88, respectively. These results indicate that the gravimetric method we employed for the calibration of $\alpha\beta$ is accurate and is within the calibration error of $\pm$10% achieved in quantitative Western blot analysis.

A subunit stoichiometry of $\alpha\beta\gamma_2$ suggests that the α and β chains of PDE may each bind one inhibitory γ subunit. This hypothesis was tested by chemical cross-linking of the subunits with dimethyl suberimidate (Figure 7), followed by Western blot analysis with subunit-specific antibodies (Figure 8). In choosing the appropriate cross-linkers, we deliberately avoided using bifunctional sulfhydryl reagents which, due to their biased selectivity, might favor cross-linking a particular pair of subunits containing many cysteine residues (Hingorani et al., 1988). This precaution is necessary because γ contains only 1 cysteine residue (Ovchinnikov et al., 1986), whereas α, and most likely β, has a total of 16 cysteine residues (Ovchinnikov et al., 1987). We also avoided using cross-linkers that alter either the negative or the positive charges of the PDE molecule. The latter is particularly important since positively charged compounds such as protamine at high concentration (Bitensky et al., 1978) or positively charged membranes (Ho & Fung, 1984) are known to stimulate the phosphodiesterase

activity. Hence, changing the net charges of PDE by the cross-linking reaction might alter the spatial arrangement of the subunits. Using dimethyl suberimidate, which satisfies the above criteria, we demonstrated that both chains of γ can be cross-linked to either α or β, but not to each other. Similar results were also obtained using glutaraldehyde (data not shown). This observation suggests that each α and β binds one γ and that the two γ chains may be spatially distant. Interestingly, with prolonged cross-linking with dimethyl suberimidate, both chains of γ can be cross-linked to either α or β (Figure 7). This result provides further support for the $\alpha\beta\gamma_2$ subunit composition of PDE.

To further correlate the result of the cross-linking and quantitative Western blot analyses to the inhibitory activity of γ, we measured the phosphodiesterase activity of trypsin-activated PDE as a function of the γ to $\alpha\beta$ ratio. We showed that total inhibition of activity was achieved with 2 mol of bound γ per mole of $\alpha\beta$ (Figures 4 and 5), suggesting that both γ subunits are inhibitory. If this is so, then results of many studies on the γ binding to activated PDE need to be reexamined more carefully. The dissociation constant for γ binding to trypsin-activated PDE has been reported to be 0.005 (Wensel & Stryer, 1986), 0.13 (Hurley & Stryer, 1982), and 15 nM (Sitaramayya et al., 1986). Although the reason for such a large discrepancy is still not known, in all these studies the dissociation constants were determined based on the assumptions that the inhibited PDE contains only one γ subunit. Evidently more refined measurement and analysis will be required to take into account the new evidence of an additional γ.

The phosphodiesterase activity of dark-adapted rod outer segment membranes can be fully activated by the addition of an excess amount of activated T_α (Fung et al., 1981). Whether or not T_α interacts directly with γ under physiological conditions, however, is not entirely clear. Kinetic studies have suggested that trypsin-activated PDE is not equivalent to light-activated PDE, which raises the possibility that transducin activates PDE by displacing, rather than removing, γ from its active site (Sitaramayya et al., 1986). On the other hand, Yamazaki et al. (1983) have reported the elution of an inhibitory factor from frog ROS with guanosine 5′-(β,γ-imidotriphosphate). Similarly, Deterre et al. (1986) have detected by ion-exchange chromatography a small population of γ-T_α-GTPγS complex in a protein preparation extracted from ROS at low ionic strength. Most recently, we have demonstrated conclusively by immunoprecipitation that γ, but not $\alpha\beta$, forms a 1:1 complex with T_α-GTPγS (Fung & Griswold-Prenner, 1989), providing convincing evidence that activated transducin stimulates phosphodiesterase activity by binding to and, most likely, physically removing γ from the catalytic $\alpha\beta$ subunits. Irrespective of the mechanism of activation of PDE, the demonstration that PDE contains two γ and that both sites must be occupied for complete inhibition of activity strongly suggests the requirement of at least two activated transducin molecules to fully stimulate one molecule of PDE in rods.

Registry No. cGMP, 9068-52-4; γ_{24-41}, 120312-97-2; γ_{44-54Y}, 125282-21-5.

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A Study of the Effect of General Anesthetics on Lipid–Protein Interactions in Acetylcholine Receptor Enriched Membranes from *Torpedo nobiliana* Using Nitroxide Spin-Labels†

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ABSTRACT: Stearic acid, phosphatidylcholine, and phosphatidylglycerol nitroxide spin-labels were used to probe the effect of 1-hexanol, urethane, diethyl ether, and ethanol on lipid–protein interactions in nicotinic acetylcholine receptor (nAcChoR) rich membranes from *Torpedo nobiliana*. For stearic acid spin-labeled at the C-14 position of the *sn*-1 acyl chain, 1-hexanol induced little change (over a wide concentration range, 0–16.7 mM) in either the ESR line shape or the proportion of motionally restricted spectral component from labels probing the protein interface. The main effect of 1-hexanol was limited to an increase in the mobility of stearic acid spin-labels probing the non-protein-associated environment. In contrast, for C-14 phosphatidylcholine spin-label, 1-hexanol decreased the fraction of spin-labels motionally restricted at the protein interface from 0.33 without 1-hexanol to 0.20 with 16.7 mM 1-hexanol, with no change in the line shape of the spectral component of these labels. The ESR spectral line shape of the fluid component due to phosphatidylcholine labels in sites away from the protein interface displayed a gradual decrease in spectral anisotropy on addition of increasing amounts of 1-hexanol. At a concentration of 1-hexanol that desensitizes half the receptors, the fraction of motionally restricted phosphatidylcholine spin-label is reduced by approximately 15%. The effect of 1-hexanol on phosphatidylglycerol spin-labels was intermediate between these two cases. Similar effects were measured with other general anesthetics, including urethane, diethyl ether, and ethanol. Thus, action at the lipid–protein interface by general anesthetics provides a plausible alternative molecular mechanism for desensitization of the nAcChoR, and possibly general anesthesia, but does not preclude some synergistic lipid disordering by anesthetics of the surrounding bilayer.

Despite much investigation, there is no consensus on the molecular mechanism of anesthesia. The wide variety of chemical substances that may produce anesthesia points to nonspecific sites able to accommodate molecules of quite different molecular architectures. It is established that the anesthetic potency of a chemical species correlates with its octanol–water partition coefficient (Franks & Lieb, 1978) and that general anesthesia can be reversed by high pressure (Lever et al., 1971). Theories diverge along two apparently incompatible lines, one suggesting that lipids of neuronal membranes are the prime site of anesthetic action and the other proposing that anesthetic action arises from direct anesthetic–protein interactions (Franks & Lieb, 1982; Miller, 1985). An alternative that has attracted little experimental attention, except in model systems (Galla & Trudell, 1981), is that the size of action is at the lipid–protein interface of excitable channels in neuronal membranes (Trudell et al., 1973; Lee, 1976; Heidmann et al., 1983; Elliot & Haydon, 1989). However, in a system containing both lipids and proteins, it is far more difficult to deduce whether the conformation of a protein is affected either by a direct anesthetic–protein interaction at the bilayer–protein interface or by motional or conformational modification of the lipids themselves which exist at the lipid–protein interface.

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