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Reciprocal Control of Retinal Rod Cyclic GMP Phosphodiesterase by Its γ Subunit and Transducin

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ABSTRACT **The switching on of the cGMP phosphodiesterase (PDE) in retinal rod outer segments by activated transducin (Tα-GTP) is a key step in visual excitation. The finding that trypsin activates PDE (αβγ) by degrading its γ subunit and the reversal of this activation by γ led to the proposal that Tα-GTP activates PDE by relieving an inhibitory constraint imposed by γ (Hurley and Stryer: *J. Biol. Chem.* 257:11094–11099, 1982). We report here studies showing that the addition of γ subunit also reverses the activation of PDE by Tα-GTP-γS. A procedure for preparing γ in high yield (50–80%) is presented. Analyses of SDS polyacrylamide gel slices confirmed that inhibitory activity resides in the γ subunit. Nanomolar γ blocks the activation of PDE by micromolar Tα-GTPγS. The degree of activation of PDE depends reciprocally on the concentrations of γ and Tα-GTPγS. γ remains bound to the disk membrane during the activation of PDE by transducin. The binding of γ to the αβ subunits of native PDE is very tight; the dissociation constant is less than 10 pM, indicating that fewer than 1 in 1,700 PDE molecules in rod outer segments are activated in the absence of Tα-GTP.**

Key words: visual excitation, rhodopsin, enzyme regulation, cyclic nucleotide cascade, G-proteins, inhibitory subunit

INTRODUCTION

The light-triggered activation of cGMP phosphodiesterase (PDE) in retinal rod outer segments (ROS) is a key step in visual excitation (Fig. 1). Photoexcited rhodopsin (R^*) catalyzes GTP-GDP exchange in transducin, a peripheral membrane protein consisting of α(39 kD), β(36 kD), and γ(8 kD) subunits. Tα-GTP, the activated form of transducin, then stimulates PDE, which rapidly hydrolyzes cGMP to close cation-specific channels in the plasma membrane (for reviews[1–4]). PDE is a peripheral membrane protein consisting of three subunits: α(88 kD), β(85 kD), and γ(11 kD).[5] A critical property of PDE is its very low catalytic activity in the dark. Limited tryptic digestion markedly increases its catalytic activity, demonstrating that PDE is subject to an inhibitory constraint.[6] Tryptic activation of PDE was found to be accompanied by degradation of its γ subunit with little change in αβ.[7] The subsequent addition of γ led to nearly complete inhibition of the catalytic activity of trypsin-activated PDE. It was proposed that PDE consists of an αβ catalytic unit that is inhibited by γ and that the role of Tα-GTP is to reverse the inhibitory effect of γ.[7,8] Interest in the mechanism of this interaction is heightened by the finding that transducin belongs to a family of signal-coupling proteins, the G-proteins, with a common structural and functional design.[9,10] G-proteins transduce many hormonal and sensory signals, as exemplified by the adenylate cyclase and phosphoinositide cascades.

Several laboratories have investigated the mechanism of activation of PDE by transducin. Yamazaki et al.[11] found that a PDE inhibitor was eluted from illuminated frog disk membranes on addition of GppNHp, a hydrolysis-resistant analog of GTP. They proposed that GppNHp-transducin pulls the inhibitor away from PDE and forms a complex with it in the cytosol. The identity of their inhibitor was not determined. Deterre et al.[12] have recently shown that bovine Tα-GTPγS forms a complex with γ but find that this complex stays on the disk membrane. They also propose that transducin-activated PDE is αβ. In contrast, Sitaramayya et al.[13] concluded from their kinetic studies that light-activated PDE consists of transducin bound to the αβ subunits of PDE, with perhaps γ also bound but displaced from its position in the inactive state. It is evident that the mechanism of light-activation of PDE is not yet unequivocally established.

We report here a series of experiments designed to answer three questions concerning the mechanism of activation of PDE.

1. Does γ participate in the physiologic regulation of PDE activity? Specifically, can γ reverse the activation of PDE by Tα-GTP?

2. What is the affinity of native PDE for γ? What fraction of PDE is active in intact ROS in the absence of Tα-GTP?

3. Is γ released into the cytosol during activation of PDE by transducin?

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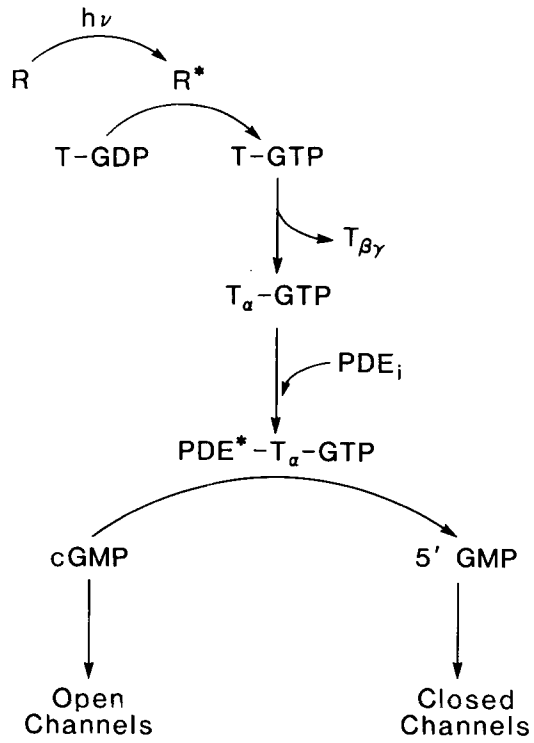

Fig. 1. Cyclic GMP cascade of vision. Abbreviations used are R, rhodopsin; R*, photoexcited rhodopsin; T, transducin; PDE_i and PDE*, inhibited and activated forms, respectively, of the cGMP phosphodiesterase. A key unresolved question is the identity of PDE*-Tα-GTP.

MATERIALS AND METHODS

Reagents

[^{35}S]GTPγS was obtained from New England Nuclear, [8-^{3}H]cGMP and 5'[U-^{14}C]GMP from Amersham, and GTPγS and GTP from Boehringer Mannheim. TPCK-treated trypsin was obtained from Worthington, soybean trypsin inhibitor and *Ophiphagus hannah* snake venom from Sigma, a polyanion SI FPLC column from Pharmacia, and frozen bovine retinas from J.A. Lawson (Lincoln, NE).

Buffers

Buffer A contained 20 mM MOPS pH 7.4, 120 mM NaCl, 30 mM KCl, 2 mM $MgCl_2$ and 1 mM DTT. For purposes of ROS or protein purification, phenylmethylsulfonyl fluoride (PMSF) was added to 0.1 mM. Buffer B (PDE radioactivity assay buffer) contained 0.1 M Tris HCl pH 8.0, 2.0 mM $MgCl_2$, 50 mM KCl, and 1 mM DTT. Buffer C (pH assay buffer) contained 20 mM MOPS pH 8.0, 0.15 M KCl, and 2 mM $MgCl_2$.

Electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli[14] with resolving gels containing 12% (w/v) acrylamide or containing a linear gradient of 8% (top) to 18% or 20% (bottom) acrylamide. The ratio of acrylamide to (N-N'-methylene) bisacrylamide was 37.5:1. Proteins were stained with Coomassie blue, and the optical density of the bands measured with a Transidyne scanning densitometer.

ROS and Protein Preparations

Rod outer segments (ROS) from frozen bovine retinas,[15] urea-stripped ROS membranes devoid of peripheral membrane proteins,[16] and transducin[17] were prepared as described previously. ROS and stripped membranes were kept in the dark and exposed to room light just before use. The dark PDE activity of ROS in the presence of 100 μM GTP was 13% of the light-stimulated activity. PDE was extracted from ROS membranes[7] and purified on a column of hydroxyapatite with a linear gradient from 0.03 M to 0.3 M sodium phosphate (pH 7.2). Fractions containing PDE (eluted at 0.13–0.17 M) were concentrated and stored in 50% glycerol at −20°C.

The γ subunit of PDE was prepared by a modification of a method described previously.[7] PDE that had been stored in 50% glycerol was twice diluted 30-fold with buffer A containing 0.02% NaN_3 and reconcentrated to remove the storage buffer. Formic acid was added to 0.1 M, and the mixture was heated at 70°C for 5 min. The pH was adjusted to 6.5 with NaOH, and the sample was centrifuged for 5 min at 30 psi in a Beckmann airfuge to remove precipitated protein. The yield of γ, determined by titrations of trypsin-activated PDE, typically ranged from 50% to 80%. Trypsin-activated PDE was prepared by treating PDE in buffer A with 65 μg/ml trypsin for 5 min at 23°C and then adding soybean trypsin inhibitor to 0.5 mg/ml. Premixed trypsin inhibitor and trypsin were found to have no effect on PDE activity at the concentrations present in the assays.

Tα-GTPγS was prepared by HPLC anion exchange chromatography with a Pharmacia Polyanion SI HR 55 column.[18] [^{35}S]GTPγ S (at a specific activity of 89 mCi per mmol) was present in the GTPγS solution used to elute Tα from bleached membranes so that the amount of functional transducin could be quantitated by a nitrocellulose filter binding assay.[15,16]

PDE Assays

PDE activity was assayed by monitoring either the release of protons with a pH electrode[19] or the production of 5'[8-^{3}H]GMP from [8-^{3}H]cGMP.[7,20] Buffer B was used for radioactive assays, and buffer C for pH assays. The course of the reaction was followed by recording the amplified signal from a pH electrode. The radioactivity assay was modified in the following way. The assays were carried out in polyethylene tubes that had been coated with a solution of 1% BSA and rinsed with distilled water (to minimize loss of protein due to surface adsorption). All components were premixed in a volume of 80 μl, and at t=0, 20 μl

of a solution containing 12 mM cGMP (4×10^5 dpm [8-^{3}H]cGMP) and 0.1 mM 5'-GMP (2×10^4 dpm 5'[U-^{14}C]GMP) was added. To terminate the assay, typically after 10 min incubation at 23°C, 200 μl of 5 mM Na_3EDTA, preheated to 95°C, was added, and the sample was placed in a boiling water bath for 4 min. The samples were cooled, treated for 30 min at 23°C with 100 μl of 1 mg/ml snake venom, and applied to a 1 ml DEAE Sephadex A-25 column (Cl^- form, equilibrated with distilled water). The column was washed with 2 ml distilled H_2O and the radioactive guanosine that was eluted was counted with 9 ml scintillation fluid. Under these conditions, the amount of 5'-GMP produced was found to be linear with time and with PDE concentration up to 2 mM cGMP hydrolyzed. Concentrations of active PDE reported here are based on a turnover number of 4200 cGMP s^{-1}.[7]

Reconstitution of Transducin-Activated PDE on Membranes

To determine the optimum PDE:R* ratio for light-activated PDE activity, PDE was incubated with different concentrations of urea-stripped ROS membranes, along with transducin and GTPγS. Maximum PDE activity was found at a ratio of 1 PDE/115 R*. Purified PDE was added to urea-stripped ROS membranes (50 μM) in ten volumes of a low-salt buffer consisting of tenfold-diluted buffer A and titrated over a period of 1–2 h with one volume of a solution of tenfold-concentrated buffer A. The membranes were incubated at room temperature for 1 h and sedimented in an airfuge (5 min, 30 psi). Supernatant and pellet were assayed for PDE activity after trypsin activation. Between 40% and 60% of the added PDE was found to be bound to the membranes. Purified Tα-GTPγS or a combination of purified transducin in the GDP form and GTPγS (10 μM) was added to the membrane-bound PDE, along with γ subunit of PDE. The membranes were incubated for 30 min at room temperature before being assayed with [^{3}H]cGMP. The PDE activity observed was expressed as a percentage of the maximal activity obtainable by trypsin activation of each sample. The transducin stock contained a trace of PDE contamination (1 PDE/8,700 transducins) as did the Tα-GTPγS (1 PDE/20,000 Tα-GTPγS).

Localization of γ Following PDE Activation

GTP (100 μM) was added to a suspension of ROS membranes in buffer C under room light, and the membranes were homogenized by being passed repeatedly through a disposable pipette tip. A portion of the membranes was assayed for PDE activity, and the remainder was immediately sedimented in an airfuge (5 min, 30 psi). The pellet was separated from the supernatant solution, washed once more with buffer C, and incubated for 1 h at room temperature to ensure complete hydrolysis of GTP bound to resid-

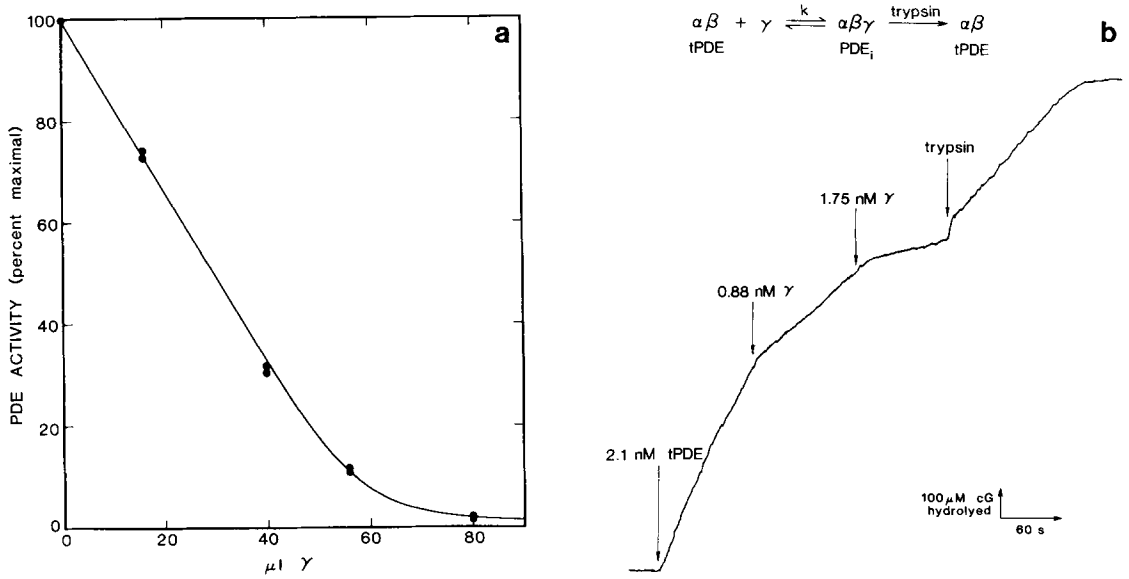

Fig. 2. Reaction of γ subunit with trypsin-activated PDE. a. Trypsin-activated PDE (0.56 nM) was assayed in the presence of increasing amounts of γ prepared by heat treatment from the same purified PDE sample used to prepare trypsin-activated PDE. Activity was measured by following the formation of [^{3}H]5'-GMP. Assays were carried out 10 min after addition of γ. The solid line represents the activity expected for a yield of 58% of that present in the holoenzyme and a K_d for the binding of γ to αβ of 5 pM. b. The kinetics of inactivation of trypsin-activated PDE by γ was measured by adding γ to PDE during the course of a proton release assay for cGMP hydrolysis.[19] The curve records the pH meter output, and so the rate of hydrolysis of cGMP is proportional to the slope.

ual transducin. The membranes were assayed again for catalytic activity to determine whether γ was released from membranes during the activation of PDE.

RESULTS

Preparation of γ and Its Interaction With Trypsin-Activated PDE

The milder conditions used here to prepare γ led to a higher yield of inhibitory activity, between 50% and 80% of that present in the native holoenzyme, compared with ~6% obtained previously.[7] Added γ inhibited PDE activity nearly stoichiometrically. The data shown in Figure 2 fit the curve calculated for a dissociation constant of 5 pM for the complex of γ with trypsin-activated PDE. The catalytic activity of trypsin-activated PDE is inhibited within 20 s following the addition of nanomolar γ (Fig. 2b), indicating that the association rate constant is greater than 5×10^7 M^{-1} s^{-1}.

Trypsin Releases From ROS Membranes

Trypsin is known to activate PDE by degrading the γ inhibitory subunit.[7] We now find that trypsin also releases PDE from disk membranes. Does release precede or follow activation? This question was answered by comparing the kinetics of appearance of PDE in the supernatant with the kinetics of enyzme activation (Fig. 3). Digestion by trypsin for 40 s released 75% of the PDE from ROS membranes but activated only 25% of the enzyme. Hence, release from the membrane precedes activation. Does trypsin only cleave the γ subunit or is the $\alpha\beta$ complex also modified by trypsin? We found that the addition of intact γ does not bring trypsin-treated $\alpha\beta$ back to the membrane though it completely inhibits its catalytic activity. In contrast, native $\alpha\beta\gamma$ solubilized at low ionic strength binds strongly to urea-stripped ROS membranes when the ionic strength is raised. The simplest interpretation of these data is that trypsin rapidly nicks the α or β subunit (or both), producing a modified $\alpha\beta$ complex lacking a segment that is essential for membrane attachment. The peptide removed from $\alpha\beta$ must be small (probably less than 1 kD), because the electrophoretic mobilities on SDS polyacrylamide gels of α and β chains exposed to trypsin are virtually the same as those of the native chains.[7]

γ Subunit Also Binds Very Tightly to $\alpha\beta$ in Native PDE

Because trypsin alters the $\alpha\beta$ complex, we wanted to determine whether the affinity of γ for native $\alpha\beta$ is like that for $\alpha\beta$ produced by trypsin treatment. The effects of dilution on the catalytic activities of membrane-bound native PDE and purified PDE in solution are shown in Figure 4. In both environments, dilution increases the specific activity of PDE, as would be expected for $\alpha\beta$ released from the inhibitory

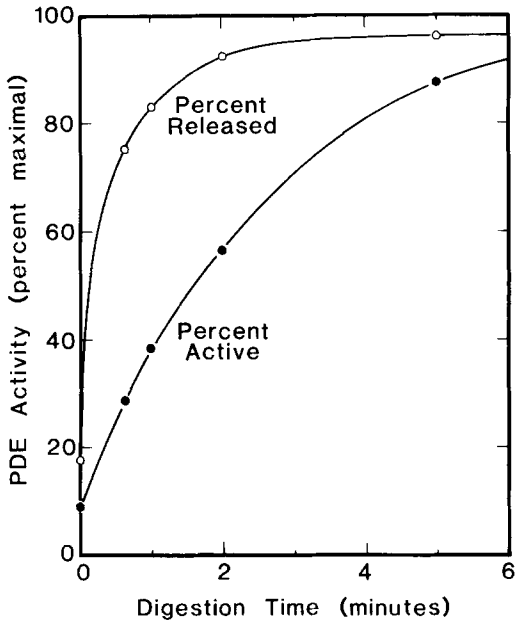

Fig. 3. Trypsin releases PDE from membranes. ROS membranes (37.8 μM R*) were incubated with TPCK-treated trypsin (0.01 mg/ml) at 23°C. At the indicated times, aliquots were removed, added to an excess of soybean trypsin inhibitor (0.05 mg/ml), and centrifuged. The pellets and supernatants were diluted and assayed for PDE activity by the pH recording method, before and after treatment with 0.1 mg/ml trypsin. Let v_1 and v_2 denote the catalytic activities of the supernatant before and after final trypsin treatment, respectively; v_3 and v_4, the corresponding ones of the pellet. The percent PDE released into the supernatant (upper curve) is given by $v_2/(v_2 + v_4)$. The percent activation of PDE (lower curve) is given by $(v_1 + v_3)/(v_2 + v_4)$.

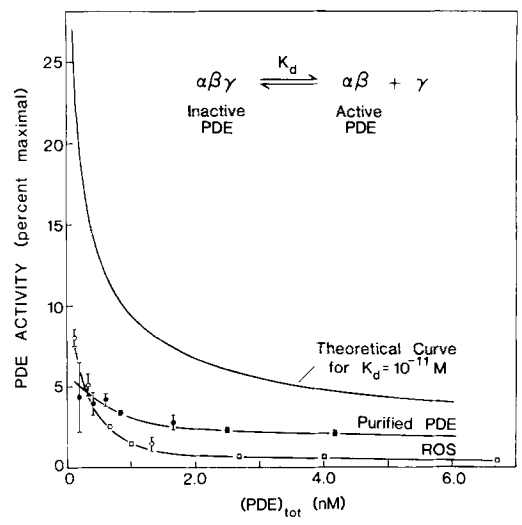

Fig. 4. The γ subunit binds very tightly to the $\alpha\beta$ subunits of native PDE. The effect of dilution on activity was measured for purified PDE (●) and whole ROS (○). Activity, measured by following the hydrolysis of [^{3}H]cGMP, is expressed as a percentage of that obtained following trypsin treatment of the same preparations. Shown for comparison (uppermost curve) is the result expected for a dissociation constant of 10 pM.

constraint of γ by dissociation of the $\alpha\beta\gamma$ complex. The dependence of specific activity on concentration indicates that the dissociation constant of the native $\alpha\beta\gamma$ is less than 10 pM (upper curve in Fig. 4). Thus, γ binds very tightly to $\alpha\beta$ in *native* PDE as it does to modified $\alpha\beta$ produced by trypsin treatment.

Nanomolar γ Subunit Reverses Light-Activated PDE Activity

The light-triggered activation of PDE by transducin in ROS can be reversed by the addition of γ subunit (Fig. 5). With no added γ, the PDE activity of ROS (3.8 μM R^*) in the presence of 10 μM GTPγS was 32% of the activity of a trypsin-activated aliquot. The K_m for cGMP under these conditions is 70 μM (data not shown). In the absence of GTPγS, the basal PDE activity of these membranes was less than 1% of the trypsin-activated level. The activation produced by light and GTPγS was nearly completely reversed by the addition of 35 nM γ subunit (inset of Fig. 5).

The equilibrium between active and inactive forms of PDE in the presence of added γ was measured by going to a lower membrane concentration (0.53 μM R^*). The total PDE concentration was 2.1 nM. With no added γ, transducin (present at 25 nM) activated 15% of the PDE. As shown in Figure 6a, the addition of 2 nMγ led to half-maximal inhibition. An apparent equilibrium constant for the inhibitory action of γ can be obtained by plotting the data as shown in Figure 6b. The concentration of free γ was assumed to be equal to the sum of the concentrations of added γ and activated PDE. A plot of $[PDE_i]/[PDE^*]$ vs. [free γ], where PDE_i and PDE^* denote the inactive and active forms of the enzyme, respectively, gives a slope of 1/(0.25 nM). This slope corresponds to an *apparent* dissociation constant of 0.25 nM for the interaction of γ with PDE^*. It is evident that γ is a potent inhibitor even in the presence of a large excess of transducin.

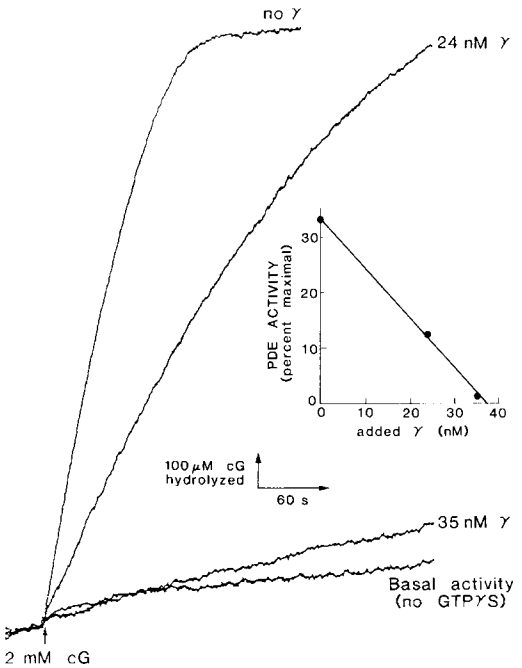

Fig. 5. Addition of γ subunit reverses light-activation of PDE. PDE in a suspension of whole ROS (R^* concentration of 3.8 μM) was activated by illumination and addition of GTPγS (10 μM). Hydrolysis of cGMP (initial concentration of 2 mM) was monitored by the pH decrease in the presence of the indicated amounts of added γ subunit. Pretreatment with trypsin abolished the inhibitory activity of γ. The inset shows a plot of PDE activity as a function of added γ.

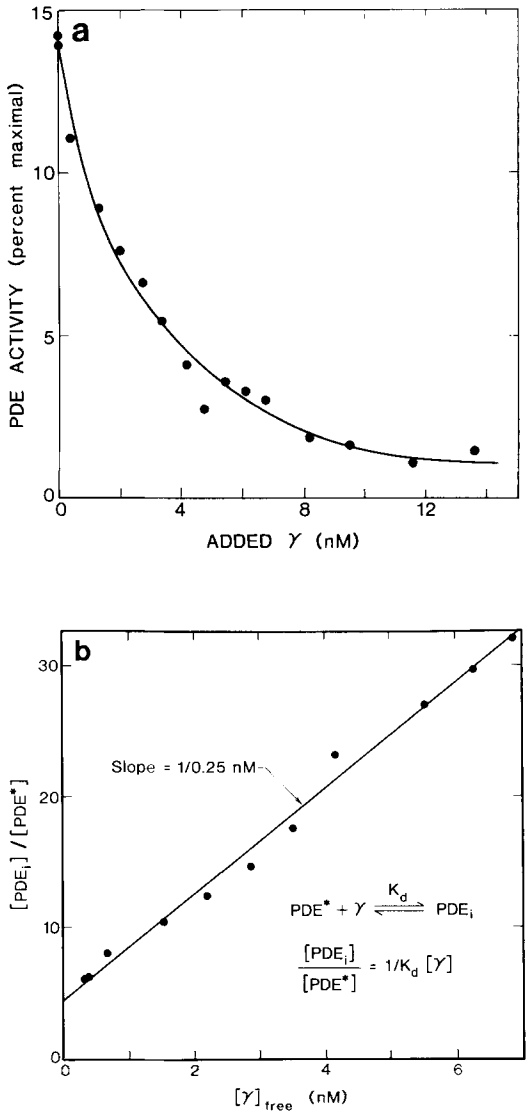

Fig. 6. Dependence of light-activated PDE activity on the concentration of added γ subunit. a. GTPγS (10 μM) was added to bleached ROS (0.53 μM R^*), along with the indicated concentrations of γ subunit of PDE. Catalytic activity was measured by following the hyrolysis of [^{3}H]cGMP. b. The ratio of inactive to active PDE ($[PDE_i]/[PDE^*]$) was determined from the maximal activity (obtained by trypsin treatment) and the level of activity induced by light and GTPγS measured at each concentration of γ.

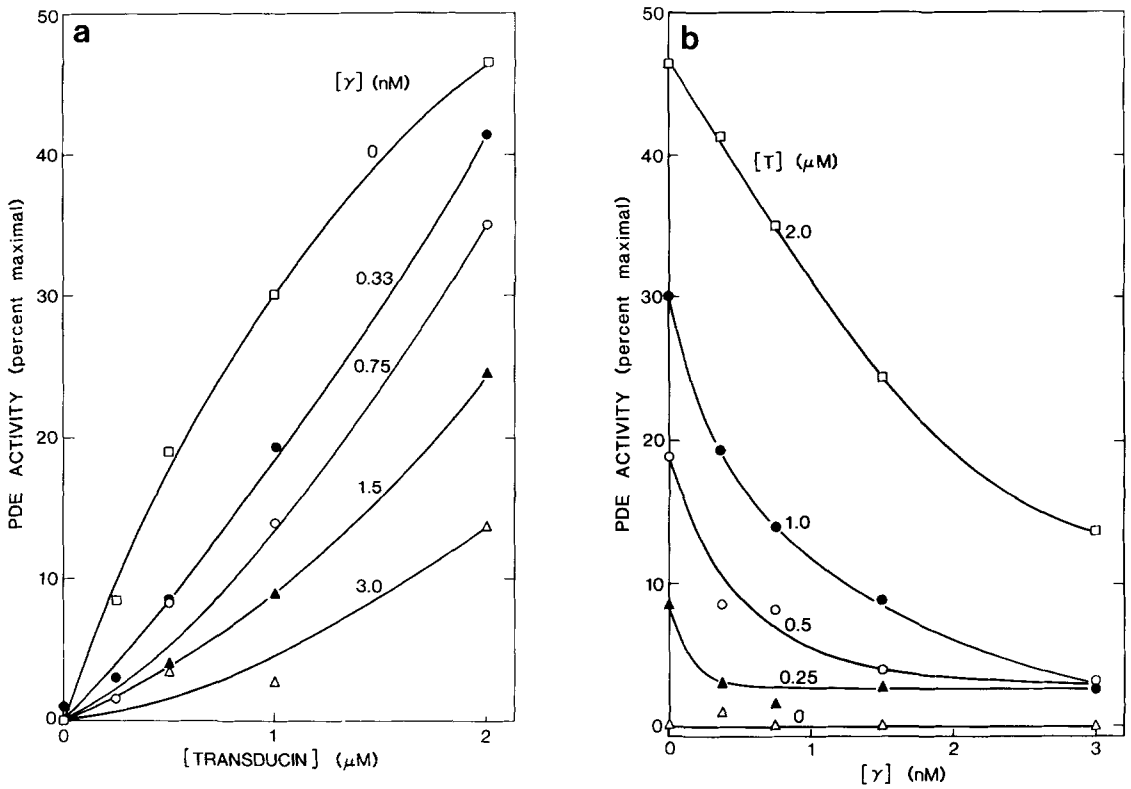

Fig. 7. Reciprocal control of PDE activity by its γ subunit and transducin. PDE activity was measured in a reconstituted system formed by adding purified PDE (0.6 nM) to urea-stripped ROS membranes (49 nM R^*). The indicated concentrations of transducin and γ were added, along with 10 μM GTPγS. Activity was measured by following the hydrolysis of [^{3}H]cGMP and is expressed as a percentage of the activity obtained by trypsin treatment.

Reciprocal Control of PDE Activity by its γ Subunit and Transducin

The regulation of PDE by the stimulatory action of transducin and the inhibitory action of γ were studied further in a reconstituted system in which the amounts of each component could be controlled. Purified PDE, transducin, GTPγS, and γ subunit were added to urea-stripped ROS membranes. As shown in Figure 7a, the concentration of transducin required to activate PDE to a particular level increases as more γ is added. For example, a threefold-higher concentration of transducin (1.8 μM vs. 0.6 μM) is needed to activate 20% of PDE in the presence of 1.5 nM added γ compared with no added γ. Conversely, more γ is needed to inhibit PDE activity as the concentration of transducin is raised (Fig. 7b). A similar reciprocal relationship between transducin and γ subunit was observed when purified Tα-GTPγS was the activator (Fig. 8). The striking finding is that micromolar transducin is required to activate PDE to a high level, whereas nanomolar γ subunit suffices to reverse this activation. Thus, activation arises from an interaction that is about 1,000-fold less strong than the one mediating inhibition of PDE.

γ Subunit Eluted From Gels Inhibits Transducin-Activated PDE

Purified PDE was electrophoresed and extracts of gel slices were analyzed for inhibitory activity to obtain further evidence concerning the nature of the inhibitor. In this gel, the 11-kD γ subunit was well resolved from 8 kD Tγ (which migrated 6 mm further) and from bromphenol blue (12 mm further). The eluates from gel slices 3 mm apart showed a single peak of inhibitory activity coinciding in position with that of the γ subunit of PDE (Fig. 9). This result, taken together with the stoichiometry of inhibition (Fig. 2), indicates that inhibitory activity resides in the γ subunit and not in a low molecular weight species present in the preparation of γ. This experiment also shows that the γ subunit renatures readily and regains its biological activity following removal of SDS.

γ Subunit Stays on Disk Membranes When PDE Is Activated

Does γ stay on disk membranes or is it released into the cytosol when PDE is activated by transducin? The activity of PDE bound to bleached membranes was

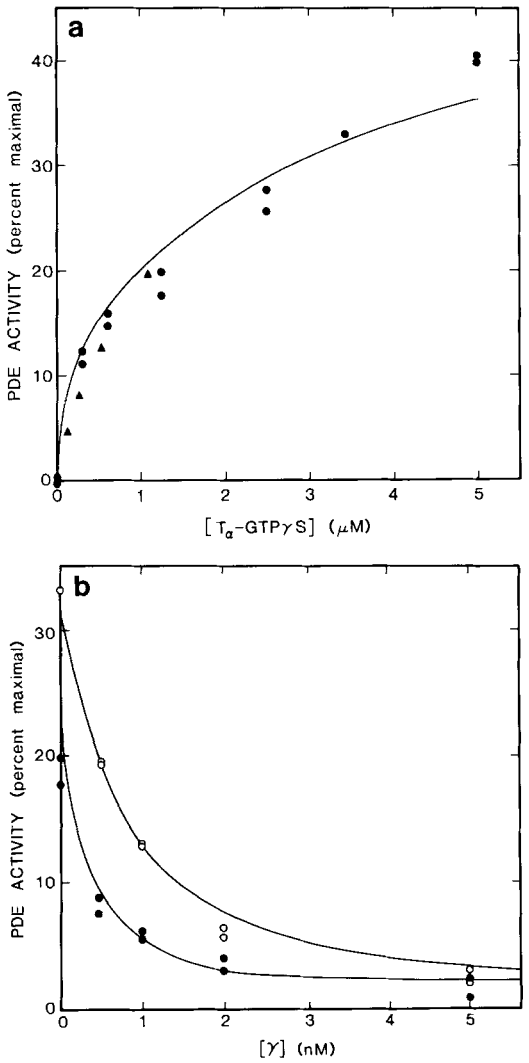

Fig. 8. Reciprocal regulation of PDE by Tα-GTPγS and γ. PDE was assayed in a reconstituted system similar to that described in Figure 6, with 195 nM R^* and 0.95 nM PDE. a. PDE activity was stimulated by addition of the indicated concentrations of Tα-GTPγS, with no added inhibitor. b. The effect of γ in this reconstituted system was determined by adding the indicated amounts to membranes containing PDE and either 3.44 μM (○) or 1.25 μM (●) Tα-GTPγS. The solid lines represent a theoretical prediction based on model 1 or 2 (Fig. 11) with an equilibrium constant for the activation reaction of 5×10^6.

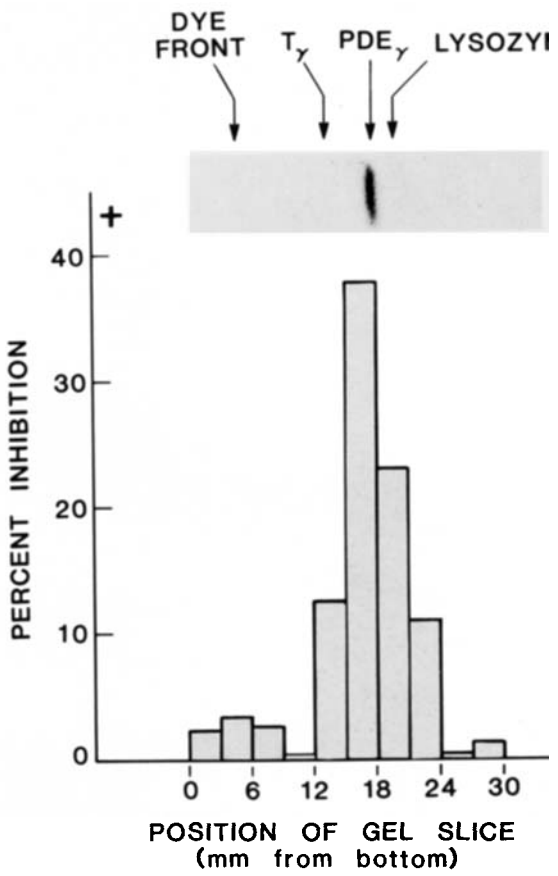

Fig. 9. Identification of γ subunit as the inhibitor of transducin-activated PDE; 40 μg of purified PDE was loaded onto an SDS-polyacrylamide gel (linear gradient, 8–20% acrylamide). Following electrophoresis, the bottom 30 mm of the PDE lane was cut into 3-mm slices. The rest of the gel, including a parallel lane that had been loaded with 10 μg of PDE, was stained with Coomassie blue; the staining pattern of this PDE lane is shown along with arrows denoting the mobility of other market proteins and the tracking dye in this gel. The unstained gel slices were crushed and shaken overnight in 200 μl of H_2O at room temperature. The soaking solution was separated from the gel slice and assayed for its ability to inhibit PDE activity in ROS activated by light and GTPγS (10 μM). The soaking solution was separated from the gel slice and assayed for the ability of 1 μl to inhibit PDE activity in a 100-μl suspension of ROS (0.48 μM R^*).

measured following addition of GTP (which activates transducin and then the PDE) and then again after washing with GTP (which removes most of the transducin). As shown in Figure 10a, PDE on the membranes returns to the inhibited state following removal of Tα-GTP, the activator. If the γ subunit of PDE had been released, it would have been washed away, resulting in a persistent activation of PDE. The observed return to the inhibited state following washing with GTP indicates that γ stayed on the membrane. Further evidence concerning the location of γ is provided by gel electrophoretic analyses of proteins released from the membranes by washing with GTPγS. As shown in the first lane of Figure 10b, the α, β, and γ chains of transducin appear in the supernatant following addition of GTPγS. The γ subunit of PDE is conspicuously absent from the supernatant. However, it can be released from the membranes by elution at low ionic strength (second lane of Fig. 10b). Densitometry of the gels showed that amount of γ eluted by GTPγS was less than 10% of that eluted from the same membranes with a subsequent low salt wash. These experiments show that nearly all of the γ subunit of PDE remains bound to disk membranes during activation by transducin.

DISCUSSION

The studies of trypsin-activated PDE by Hurley and Stryer[7] showed that activation can be reversed by addition of γ and strongly suggested that Tα-GTP

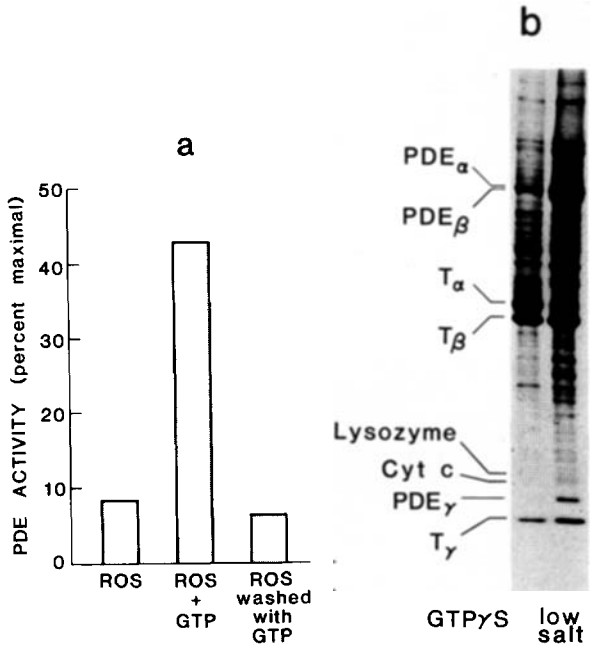

Fig. 10. Activation by Tα-GTPγS does not release inhibitor from membranes. a. Histogram displaying relative PDE activity measured in bleached ROS membranes under the following conditions: (1) in the absence of GTP; (2) in the presence of 100 μM GTP; and (3) after washing with 100 μM GTP, removal of excess GTP, and 1-h incubation (to allow for hydrolysis of GTP bound to residual transducin). Total PDE activity was determined by trypsin treatment. b. Protein staining patterns of SDS polyacrylamide gels of ROS supernatants: (1) proteins eluted with 5 μM GTPγS and moderate salt; and (2) proteins eluted with distilled water after first washing with 5 μM GTPγS and moderate salt.

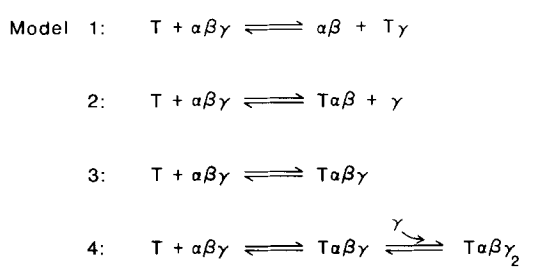

Fig. 11. Postulated molecular mechanisms for the activation of phosphodiesterase by transducin. T denotes Tα-GTP, αβ denotes the catalytic subunits of PDE, and γ its inhibitory subunit; αβ and γ remain bound to the disk membrane during activation of PDE.

activates the enzyme by reversing the inhibitory action of γ. The experiments reported here demonstrate that γ can reverse the activating effect of Tα-GTPγS. Hence, γ is central to the regulation of PDE in light-activation. As shown in Figures 6 and 7, γ is a potent inhibitor of transducin-activated PDE. Nanomolar concentrations of γ antagonize PDE activation by micromolar concentrations of Tα-GTPγS. The identity of γ as the inhibitor of transducin-activated PDE is established by the finding that inhibitory activity electrophoretically migrates together with γ on SDS-polyacrylamide gels (Fig. 9). The high yield of inhibitory activity, between 50% and 80% of that present in the holoenzyme, obtained by our milder procedure, reinforces the assignment of γ as the inhibitory component of PDE.

Several models for the mechanism of activation of PDE by transducin can be envisaged (Fig. 11). The initial step in each model is the binding of Tα-GTP (abbreviated as T) to the PDE holoenzyme (αβγ). The dissociation of γ from αβγ is much too slow to be pertinent to the activation of PDE by transducin.[7] Tα-GTP must first attack αβγ. In model 1, T carries γ away from the holoenzyme, leaving an active αβ complex. In model 2, T displaces γ from the holoenzyme, producing an active Tαβ complex. In model 3, T forms a complex with the holoenzyme, altering the interaction of γ with the catalytic αβ unit. Our finding that γ reverses the activation of PDE by T clearly rules out model 3. However, our data are consistent with a modified version, model 4, in which added γ binds to a second site to form an inhibited $T\alpha\beta\gamma_2$ complex.

Models 1 and 2 are the simplest ones compatible with our experimental findings. In model 2, γ reverses activation by driving the equilibrium back to the inhibited αβγ form. In model 1, γ inhibits by combining with αβ. Model 1 is supported by the recent work of Deterre et al.[12] that shows that a complex of Tα-GTPγS and γ can be released from activated disk membranes by washing them with a low ionic strength medium. The work of Yamazaki et al.[11] also supports model 1. However, they found that inhibitory activity is released from activated frog disk membranes at physiologic ionic strength, contrary to our result (Fig. 10) and that of Deterre et al.[12] on bovine disks. Models 2 and 3 are favored by Sitaramayya et al.[13] on the basis of their finding that light-activated PDE has a much higher K_m for cGMP than does trypsin-activated PDE (about 1 mM compared with 140 μM). They also observed that a much higher concentration of γ was needed to inhibit light-activated PDE than to inhibit trypsin-activated PDE. In our system, the K_m of trypsin-activated and light-activated PDE for cGMP are nearly the same, about 70 μM, as previously measured by several laboratories.[5-7,21] It should also be noted that trypsin-activated PDE, in contrast with light-activated PDE, is not bound to the disk membrane (Fig. 2), and so it need not have the same properties as αβ formed according to model 2 by the carrying away of γ by T. Indeed, the interaction of T with αβ or with γ must change the concentration dependence of inhibition by γ for activation to occur. In summary, we favor model 1 but do not exclude models 2 and 4 from further consideration. The data shown in Figures 7 and 8 do not discriminate between these models. It is evident that new experimental approaches, such as fluorescence energy transfer and chemical crosslinking to establish proximity relationships, are needed to unravel the molecular mechanism of activation of this key enzyme. An intriguing possibility is that modification of the interaction between transducin and the phosphodiesterase is at the heart of adaptation. If so, then the conflicting findings of different laboratories

may be an expression of different initial states of the systems studied.

Our finding that micromolar levels of transducin are needed for substantial PDE activation agrees with the results of previous studies of dark-adapted ROS,[15] urea-stripped ROS membranes, and reconstituted membranes.[22-24] Likewise, the PDE activity of illuminated ROS in the presence of GTPγS can be increased beyond the level achieved with endogenous transducin by the addition of micromolar transducin (unpublished observations). It is noteworthy that 3 nM endogenous transducin can give rise to 15% activation of PDE.[25] Liebman and Sitaramayya also noted a difference between the effects of endogenous transducin and added transducin[26]. Likewise, the T-γ complex isolated by Deterre et al.[12] is sufficiently stable to be isolated by chromatography, yet the amount obtained is less than half of that expected on the basis of the concentrations of transducin and PDE. These observations suggest that there are two classes of transducins; identifying the source of the heterogeneity of the transducin-PDE interaction may be essential for gaining a full understanding of activation and adaptation.

Native PDE in solution or bound to disk membranes has very high affinity for its inhibitory γ subunit. The dissociation constant K_d is less than 10 pM (Fig. 4). The fraction f of active PDE at a total PDE concentration c is given by $f^2/(1-f)=K_d/c$. In intact ROS, the concentration of PDE is about 30 μM, and so $f < 5.8 \times 10^{-4}$. Thus, fewer than 1 in 1,700 PDE molecules are catalytically active in situ in the absence of activated transducin. This low degree of activity of PDE in the dark state is essential in enabling rods to detect single photons and in keeping unproductive hydrolysis of cGMP at a tolerable level in terms of the energy required to resynthesize cGMP. Activated PDE has a k_{cat}/K_m value of about 7×10^7 M^{-1} s^{-1}. Fully activated PDE at a concentration of 30 μM would hydrolyze cGMP with a 1/e time of 0.5 ms (for $[cGMP] << K_m$). Hence, a dissociation constant of <10 pM implies that cGMP hydrolysis time in the absence of activated transducin is longer than 0.8 s, consistent with ^{18}O studies showing that the turnover time of cGMP in the dark in about 1.5 s[27]. The interaction of γ with αβ is strong enough to keep the enzyme in a highly inhibited state in the dark, yet not so strong as to preclude rapid activation by transducin.

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