Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 2424–2428, April 1988
Biochemistry

cGMP phosphodiesterase of retinal rods is regulated by two inhibitory subunits

(phototransduction/G protein)

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Communicated by Lubert Stryer, November 12, 1987 (received for review September 1, 1987)

ABSTRACT **The cGMP phosphodiesterase (PDE) of cattle retinal rod outer segments comprises three types of subunits: the two heavy catalytic ones, PDEα and PDEβ, each around 85 kDa, and the light inhibitory one, PDEγ or I (11 kDa). The relative stoichiometry is usually assumed to be 1:1:1. PDE activation in the visual transduction cascade results from removal of the inhibitor by the α subunit of transducin (Tα). The stoichiometric complex Tα-I, separated from activated PDE, has been isolated and characterized. Analyzing now the activated PDE, we find that it still contains some inhibitor and is resolvable into two species, one with 50% of the inhibitor content of the native enzyme and the other totally devoid of it. The same two species are observed upon activation of PDE by very short tryptic proteolysis, which specifically degrades the inhibitor. This leads us to conclude that the composition of the native enzyme is PDEαβ-I_2. The two inhibitory subunits are differentially bound, sequentially removable, and exchangeable between the native complex PDEαβ-I_2 and the fully active PDEαβ. The possibility of this exchange precludes as yet an unambiguous estimate of the actual activity of the intermediate complex PDEαβ-I. The differential binding and the exchangeability of the inhibitors raises the possibility of a fast, diffusion controlled, switch-off mechanism of PDE activity after a flash, which would shortcut the inactivation resulting from the slow GTPase rate of transducin.**

In vertebrate retinal rod outer segments (ROS), the photoreception process leading from photoexcitation of rhodopsin to hyperpolarization of the cell membrane is mediated by a rapid cGMP hydrolysis (1–3). The cGMP phosphodiesterase (PDE) responsible for this activity is a multisubunit protein, peripherally attached to the disc membrane, consisting of a heterodimeric catalytic complex: PDEα (90 kDa) and PDEβ (85 kDa), associated with at least one inhibitory subunit, subunit I (11 kDa) (4, 5). This PDE can be activated by trypsin (6), which proteolyzes subunit I and relieves the activity of the catalytic complex PDEαβ. The inhibitor has been purified and shown to be able to inhibit the activity of purified PDEαβ (7). Studies and analysis of the regulation of activity of trypsin-activated PDEαβ by purified inhibitor have been performed under the simplest assumption: that a single inhibitory subunit blocked the activity of the catalytic complex (8, 9).

The natural activator of PDE is transducin (T), the G protein specific to retinal rods (10–12). As for other G proteins, upon the GDP/GTP exchange catalyzed here by photoexcited rhodopsin, the Tα–GTP (39 kDa) subunit dissociates from Tβγ (37 kDa + 6 kDa). Tα is released from the membrane into the solution, diffuses to interact with the PDE complex, and relieves the inhibition of PDE activity. We have recently demonstrated (13) that permanently activated Tα–guanosine 5′-[γ-thio]triphosphate (GTP[γS]) binds the PDE inhibitor and forms a membrane-bound stoichiometric Tα-I complex physically dissociated from the activated PDE. Extending our work to purification of this activated PDE, we have separated two different active PDE species, one of them still containing about one-half of the inhibitor content found in the inactive PDE and the other totally devoid of inhibitor. Similar results were obtained upon limited proteolytic activation of PDE, leading us to propose here that the inactive PDE complex includes two inhibitory subunits. This opens possibilities of subtle regulation processes of PDE activity by transducin.

MATERIALS AND METHODS

Sample Preparation. ROS membranes were prepared as described (14) and preserved at −80°C. The thawed pellets were homogenized, illuminated, and then washed in medium salt buffer, to eliminate some minor proteins not relevant to this study. Extraction and "crude" purification were then carried out using the light, nucleotide, and ionic-strength dependence of binding of the species (14). Crude PDE was obtained by low ionic strength extraction of the illuminated membrane pellet and crude transducin was obtained by subsequent extraction of the same pellet after addition of 100 μM GTP[γS]. Total extract was obtained by direct low ionic strength extraction of an illuminated membrane pellet in the presence of GTP[γS]. Low salt buffer was 5 mM Hepes/1 mM dithiothreitol, pH 7.5; medium salt buffer was 20 mM Hepes/120 mM KCl/1 mM dithiothreitol, pH 7.5. Rhodopsin concentration in all extraction procedures was 2 mg/ml. GTP[γS] was used at 100 μM.

Protein Chromatography. Separation and purification of transducin subunits, native PDE, and the various PDE units were performed as described (15), on an ion exchange column (Polyanion SI from Pharmacia) (unfortunately this very efficient column is not commercially available anymore, but we have a stock of them). Elution was obtained by Na_2SO_4 gradients (from 0 to 600 mM) in a buffer containing 20 mM Hepes, pH 7.5/1 mM $MgSO_4$/5 mM 2-mercaptoethanol. Various gradient programs were used to optimize the separation of the different PDE species (see figure legends). Proteins were eluted from the column (0.5 ml/min) followed by UV absorption. Fractions (250 μl) were collected.

Protein Titration. The protein content of crude extracts, of purified solutions, or of fractions eluted from the column were estimated through the Coomassie blue colorimetric assay with bovine serum albumin as a standard. For analysis of elution fractions $NaDodSO_4$/PAGE was performed on 16% acrylamide gels (16). Gels were stained with Coomassie

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Abbreviations: ROS, rod outer segments; T, transducin; PDE, retinal cGMP phosphodiesterase; GTP[γS], guanosine 5′-[γ-thio]-triphosphate.

blue, and estimates of the relative protein content were derived from densitometry of the gels. (See text and Fig. 2 legend for linearity check.)

PDE Activity Assays. PDE activity of the elution fractions was measured by the pH metric method (17) at 20°C. Elution fractions (50 μl) were added to 350 μl of a medium containing: 20 mM Hepes, pH 7.5/2 mM $MgCl_2$/500 mM KCl. The PDE velocity was determined after adding 7.5 μl of 25 mM cGMP. Under such conditions, in the absence of ROS membrane, the turnover rate of PDE fully activated by trypsinization was $\approx$1000 mol of cGMP per sec per mol of PDE.

RESULTS

PDE Activated by Tα–GTP[γS] Is Separable in Two Species That Differ in Inhibitor Content and Specific Activity. When retinal rod membranes have been fully illuminated, if GTP is suppressed from the medium, transducin binds quantitatively to photoexcited rhodopsins on the discs (11). Crude PDE (80% pure) can then be extracted from the membrane pellet by low ionic strength washing. This PDE has a very low basal activity. On gel filtration column and our Pharmacia FPLC Polyanion exchange column, it elutes as a single peak (Fig. 1*A*). $NaDodSO_4$/PAGE of the peak fractions shows that it contains the three types of subunits—PDEα, PDEβ, and I. The two larger subunits, which have nearly identical molecular masses and are very closely related (4), are present in equal molecular amounts, but the relative stoichiometry of the very small inhibitor subunit cannot be reliably estimated from densitometry of the gel fractions.

When, after the extraction of PDE, GTP[γS] is added to the illuminated ROS membrane pellet, transducin is activated and released from R*. It can be extracted quantitatively from the membrane pellet (crude transducin) by low ionic strength washing. On the ion exchange column, this active transducin elutes as two peaks corresponding to the separated subunits Tα–GTP[γS] and Tβγ (Fig. 1*B*).

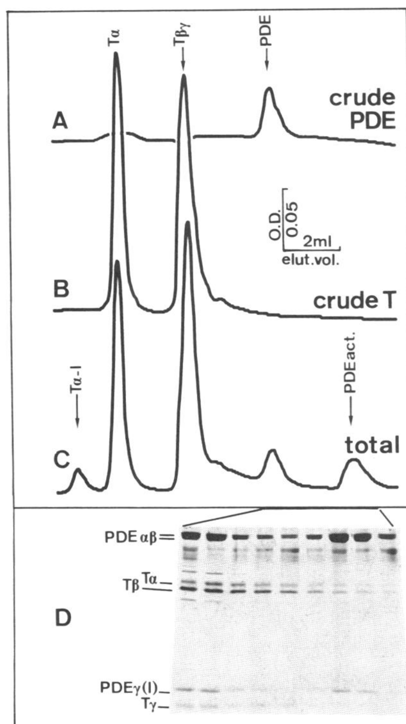

FIG. 1. Separation of activated from inactive PDE by ion exchange chromatography. (*A–C*) Elutions by a Na_2SO_4 gradient of various ROS extracts loaded on a Pharmacia FPLC Polyanion column. Each extract was obtained from aliquots of ROS membrane suspensions containing 5 mg of rhodopsin. The gradient slope is 8.8 mM/ml from 0 to 130 mM and 16.6 mM/ml above 130 mM, and the elution rate is 0.5 ml/min. The profile is shown between 50 mM and 400 mM Na_2SO_4. (*D*) $NaDodSO_4$/polyacrylamide gel of fractions from the indicated region.

But if GTP[γS] is added to the illuminated membranes before the low ionic strength extraction, transducin interacts with PDE before both proteins are extracted. On the ion exchange elution profile of this total extract, a supplementary peak is then found to precede the Tα–GTP[γS] and Tβγ peaks (Fig. 1*C*). We previously characterized this peak (13) as due to the formation of a stoichiometric complex Tα-I, which is dissociated from the activated PDE complex. The dissociation is not an artifact because of strong interactions with the charges of the ion exchange resin: on a gel filtration column, part of the inhibitor is also dissociated from the heavy catalytic complex of the PDE (13) and migrates with an apparent molecular mass around 50 kDa, close to that of Tα. This represents most probably the Tα–I complex that is not resolved from free Tα on this column.

Under the usual assumption of 1:1:1 relative stoichiometry of the α, β, and I subunits in PDE, one would expect the activated PDE resulting from the removal of inhibitor by Tα to be devoid of subunit I. We wondered whether this activated PDE could be separated from the native one. Indeed, we found on the ion exchange elution profile of the GTP[γS]-activated total extract, in addition to the peak corresponding to the inactivated PDE of the crude extracts, a second PDE peak that eluted at higher salt concentration. To our surprise, this peak contained, besides the PDEα and PDEβ catalytic units, a significant amount of inhibitory subunit (Fig. 1*D*). But, after slightly modifying the gradient program and loading the column with total extracts from larger membrane samples (>10 mg of rhodopsin), we noticed that the second peak could be resolved into two partially overlapping components (Fig. 2*A*): a major one in which the ratio I/PDEαβ was about one-half of that measured in the inactive PDE peak, and a minor one that appeared totally devoid of I (Fig. 2*C*). As the small inhibitor stains much less than the catalytic subunits, the quantitation of inhibitor staining and its normalization to that of PDEαβ was based on the scanning of many gels with different loads of the same fractions. The total protein content of the elution fractions was also independently estimated by the Coomassie blue colorimetric method in solution, and this calibration was used to normalize the loads of the fractions compared on gels. This complete procedure was repeated on three preparations, and partial checks were made on three others. The ratio I/PDEαβ in the major PDE peak resulting from the action of Tα–GTP[γS] was constantly found to lie between 40% and 60% of that found for the inactive PDE peak in the same preparation.

Measurements of the cGMP phosphodiesterase activity of the peak fractions indicated that both components of the new peak corresponded to activated PDE and, indeed, resolved these components better than did the UV elution profile (Fig. 2*A*). The specific activity was more than twice as high for the PDE that is totally devoid of inhibitory subunit than for that of the first component. The simplest interpretation for the existence of three PDE peaks with 2:1:0 relative amounts of inhibitor for a normalized amount of PDEαβ is that they correspond, respectively, to inactive PDE with two inhibitory subunits and to two different states of activated PDE with, respectively, one and zero inhibitory subunit. We thus denote the three PDE peaks as PDEαβ-I_2 (inactive), PDEαβ-I, and PDEαβ.

Further experiments were performed, first by incubating crude PDE with crude transducin solutions in the absence of membranes and then by incubating purified inactive PDE with purified Tα–GTP[γS], separated from Tβγ. The same qualitative pattern of PDE eluting in three peaks was always found, but the relative amounts of PDE in the different peaks

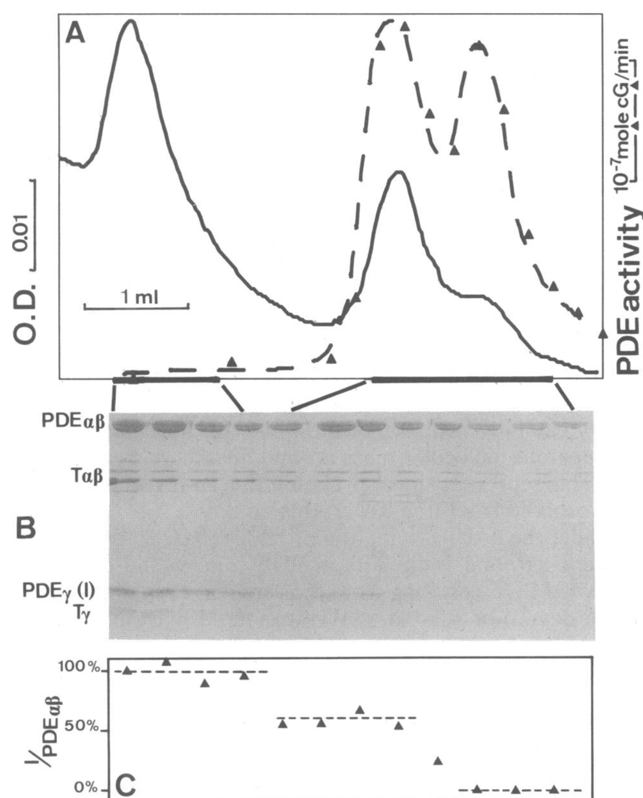

FIG. 2. Analysis by ion exchange chromatography and characterization of the three different states of PDE obtained upon activation by Tα–GTP[γS]. A total extract was prepared from a suspension of ROS membranes containing 11 mg of rhodopsin and was chromatographed. (*A*) Elution profile (—) and cGMP hydrolysis activity of the elution fractions (----). The Na_2SO_4 gradient slope is 16.6 mM/ml from 0 to 180 mM and 53 mM/ml above 180 mM. The profile is shown between 150 and 400 mM Na_2SO_4. (*B*) $NaDodSO_4$/PAGE of underlined fractions (80 μl per well). The bands around 30 kDa are from an unknown contaminant also present in crude inactive PDE preparations. (*C*) Densitometry of the Coomassie blue-stained gel. To get reliable above-background gel stain detection of the weak inhibitor band, this gel had to be overloaded for the PDEαβ doublet. Linearity of the densitometric scan of the subunit I band was checked by varying the gel load by factors of 2 on specific fractions. Densitometry of the PDEαβ doublet was performed on another gel loaded with only 10 μl per well of the same elution fractions. The ratio of the optical densities of subunit I and PDEαβ bands is arbitrarily normalized to 100% for the first PDE peak.

depended on the contents of Tα–GTP[γS] and PDEαβ-I_2 in the initial mixture. However, even with a ratio of 100 Tα per PDEαβ-I_2 in the mixture before elution, the stripping of subunit I from PDEαβ-I_2 was far from complete, and the PDEαβ peak remained much smaller than PDEαβ-I (Fig. 3). As discussed later, the yield of stripping of inhibitor from PDE might depend on transducin concentration rather than on its stoichiometry with respect to native PDE.

Analogies Between Tα–GTP[γS]-Activated PDE and PDE Activated by Short Proteolysis. Trypsin activates PDE by degrading the inhibitory subunit (6, 7). We first observed that after the usual treatment of crude PDE by trypsin (9) the activated enzyme eluted on the ion exchange column as a single peak, totally devoid of inhibitor. Its position coincided with that of the minor peak, devoid of inhibitor, observed after GTP[γS] activation, and its specific activity (V_{max}, ≈1000 cGMP hydrolyzed per sec per PDE molecule) was very close to that measured previously for this minor peak. We then analyzed the progressive effect of mild tryptic digestion at 0°C (Fig. 4): upon a very short digestion time, the native PDE peak decreases first to give rise to a major

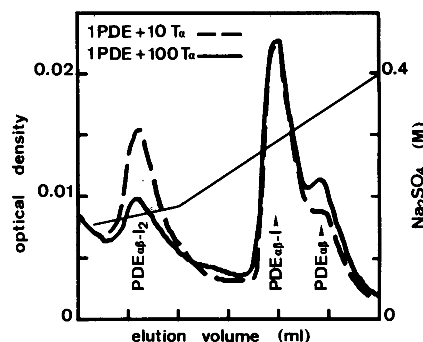

FIG. 3. Activation of purified PDE by purified Tα–GTP[γS]. The elution profiles were obtained after loading on the column solutions containing 0.04 μM PDE + 0.4 μM Tα–GTP[γS] (----) and 0.04 μM PDE + 4 μM Tα–GTP[γS] (—). The increase on the left is due to the tail of the Tα peak.

peak that elutes at the same ionic strength as the PDEαβ-I peak observed previously with Tα–GTP[γS] and a minor proportion of PDE totally devoid of inhibitor. Only upon longer digestion does the native PDE peak disappear completely, then the PDEαβ-I peak decreases on its turn to be replaced quantitatively by the peak devoid of inhibitor. This later peak is fairly resistant to further proteolysis: it is not significantly degraded after 20 min of the same treatment (at 0°C). Densitometry of the gels of the various elution fractions confirmed that the relative inhibitor content in the three peaks obtained upon proteolysis are in the same 2:1:0 ratio as obtained for transducin-activated PDE. The specific cGMP phosphodiesterase activities of the two trypsinized PDE species are, within the accuracy of our calibrations, identical to that found for the corresponding transducin-activated species (compare activity data in Figs. 2 and 4). But, by contrast with transducin-induced activation, which under our conditions always produced only a small proportion of totally stripped PDEαβ, tryptic activation leads easily to nearly pure PDEαβ.

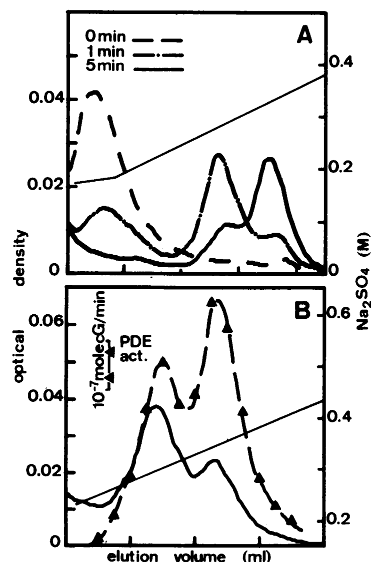

FIG. 4. Activation of PDE by progressive trypsinization. (*A*) Elution profiles of 1-ml aliquots of crude PDE extracts (containing 80 μg of PDE) that were proteolyzed by trypsin (40 μg of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin) for various times at 0°C (sample in melting ice). The proteolysis was blocked by adding 200 μg of soybean trypsin inhibitor. ----, 0 min (inhibitor added before trypsin); -·-, 1 min; —, 5 min. (*B*) Elution profile of a 2-ml crude PDE extract proteolyzed for 3 min (same conditions as in *A*). —, Optical density; ----, cGMP hydrolysis activity of the eluted fractions.

Exchange of Inhibitor Subunit Between the Native and the Trypsin-Activated States of PDE. Aliquots of native PDEαβ-I_2 and fully stripped PDEαβ prepared by trypsinization were loaded and eluted separately on the ion exchange column. The elution profiles were compared to that obtained after loading on the column the same amount of the two components mixed together (Fig. 5). One sees in the elution profile of the mixture a diminution of the two original component peaks and the formation of a PDEαβ-I peak that was absent from the individual component profiles. This can only result from an exchange reaction as follows: PDEαβ-I_2 + PDEαβ ⇋ 2 PDEαβ-I.

DISCUSSION

Evidence for Two Inhibitory Subunits per Native PDE Complex. The common assumption that the retinal rod cGMP phosphodiesterase is composed of two nearly identical catalytic subunits and a single inhibitory subunit had never been carefully scrutinized. While fair evidence existed that the two catalytic subunits form an indissociable 1:1 complex, the relative stoichiometry of the inhibitory subunit in the native enzyme remained uncertain, due mostly to its small molecular mass. It was just the simplest hypothesis to assume that there would be one inhibitor per complex.

We have strong evidence that there are at least two inhibitory subunits per complex and, most probably, only two. In the elution pattern of the proteolyzed enzyme, the first supplementary peak is well resolved from the peak of native PDE and the I/PDEαβ ratio in this first proteolyzed peak is exactly one-half that found in the peak of native PDE. Even upon the shortest and mildest proteolysis, no intermediate peak could ever be detected between the native PDE peak and this PDEαβ-I peak, nor any continuous backgound of PDE subunits. Phosphodiesterase activity measurements on all the elution fractions confirm also that no activity is observed outside the two peaks corresponding to PDEαβ-I and PDEαβ. As one does not expect trypsin to proteolyze more than one inhibitory subunit at a time, it is most probable that the three peaks containing PDEαβ correspond, respectively, to PDEαβ-I_2 (native inactive PDE), PDEαβ-I, and PDEαβ. In their early study of proteolytic activation of PDE, Hurley and Stryer (7) compared the sizes of native and trypsin-activated PDE by gel filtration. From their data and their calibration, one can estimate that trypsin-activated PDE is lighter than the native one by ≈25 kDa: although the accuracy of such determinations should not be overestimated, this difference corresponds better to two 11-kDa inhibitory subunits being stripped rather than one.

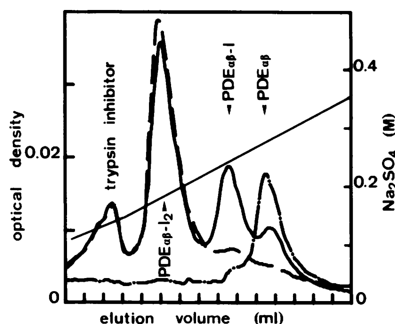

FIG. 5. Evidence for the exchange of inhibitor subunits between PDEαβ-I_2 and PDEαβ. PDEαβ was prepared by 5-min proteolysis (same conditions as in Fig. 4*A*) of a 3.5-ml sample of crude PDE (80 μg of PDE per ml) and purified by a first passage on the ion exchange column. This purification eliminates the inhibited trypsin and excess inhibitor, which elute at much lower ionic strength. The purified PDEαβ was divided in two aliquots and the fractions neighboring this peak were preserved. In another 3.5-ml sample of the same crude PDE preparation, trypsin inhibitor was added (20 μg/ml), but not trypsin, and the sample was divided in two PDEαβ-I_2 aliquots. The elution profiles shown were obtained by eluting successively on the same column: —, a mixture of one aliquot of PDE1β-I2 and one aliquot of PDEαβ; –·–, one aliquot of PDEαβ and the same volume of extraction buffer; ---, one aliquot of PDEαβ-I2 and the same volume of the fractions that had eluted just above and below the PDEαβ peak in the first purification step of PDEαβ. One sees in the elution profile of the mixture PDEαβ-I_2/PDEαβ a PDEαβ-I peak that is created at the expense of both components of the mixture, most likely by exchange of PDE inhibitor between PDEαβ-I_2 and PDEαβ.

Comparison of PDE Activation by Tα–GTP[γS] or by Trypsin May Give Clues to the Natural Activation Mechanism. *In vitro*, on broken ROS suspensions, a puzzling difference is observed between the PDE activity obtainable by trypsin action and that obtained by illumination in the presence of GTP[γS] (8, 12). The V_{max} for cGMP hydrolysis is usually 3–5 times higher for the trypsin activated PDE. This might be due to proteolytic modifications of the catalytic complex that would increase the specific activity. We demonstrate that both the proteolytic and the natural activation process (simulated here by the action of GTP[γS]) generate the same two functional states of PDE. The difference in specific activities between the two types of preparations is only due to differences in the relative amounts of PDEαβ-I and PDEαβ generated by the two activation processes. The low specific activity observed *in vitro* upon transducin activation is due to the limited proportion of fully stripped PDEαβ obtained, rather than to a lower cGMP turnover rate of this native PDEαβ. Wensel and Stryer (9) had observed that the fully proteolyzed enzyme becomes soluble and does not reattach to ROS membranes even when reinactivated by excess inhibitor. This solubilization must then result from a small proteolytic cleavage of the PDE catalytic complex that modifies the membrane attachment but not the kinetics characteristics of the catalytic complex.

The observation that the active states of PDE generated by proteolysis are kinetically identical to that obtained by interaction with transducin strongly supports the concept that transducin activates PDE by removing the inhibitory subunits from the holoenzyme. The two steps observed to reach the fully active PDEαβ indicate that the inhibitory subunits are taken off one at a time, which further confirms our suggestion (13) that one Tα–GTP[γS] binds stoichiometrically to one inhibitory subunit and physically dissociates it from the catalytic complex. The two inhibitory subunits may have different binding affinities with the catalytic complex of the PDE. Our method of separating the complexes on an ion exchange column does not allow us to infer the exact values of the binding constants of the subunits from the elution patterns obtained, as dissociations or recombinations may occur in the column during the loading and/or the elution processes. We observed, however, that the concentration of transducin in the mixture before elution influences the relative yields of the two active species (Fig. 3).

High affinities of the PDE inhibitors for the catalytic complex are required for perfect inhibition of the native PDE. Quantitative inhibition of trypsin-activated PDE by purified inhibitor (8, 9), when analyzed with the (now clearly inexact) assumption that one subunit I inhibits totally one PDEαβ complex, gave an affinity of the order of 10^{-9} to 10^{-11} M for the binding of this inhibitor to the catalytic complex. This may be taken as the best estimate for the binding constant of the first inhibitor to the fully stripped catalytic complex: PDEαβ-I ⇋ PDEαβ + I. Kinetics arguments show that transducin cannot physiologically activate PDE to the fully stripped state PDEαβ simply by binding to free inhibitor in the above equilibrium: as the binding rate constant for proteins in solution is diffusion limited around 10^8 s^{-1}·M^{-1} (18), the dissociation rate constant of I from PDE-I is at maximum in the range of 10^{-1} to 10^{-3} sec^{-1},

which means a lifetime of the order of 10 to 1000 sec for the bound state. The response time of PDE activation by transducin is <100 msec (1). This stage of activation must therefore proceed through the formation of an intermediate state Tα-I-PDEαβ in which transducin binds to the inhibitor in the native PDE complex and modifies its conformation to a state I*, which has a lower affinity for the catalytic complex, so as to allow its fast dissociation, according to the scheme: $T\alpha + \text{I-PDE}\alpha\beta \overset{K_d}{\rightleftharpoons} T\alpha\text{-I-PDE}\alpha\beta \rightleftharpoons T\alpha\text{-I*-PDE}\alpha\beta \rightleftharpoons T\alpha\text{-I*} + \text{PDE}\alpha\beta$. The first binding step implies that the rate of activation of PDE, which depends on the ratio $[T\alpha\text{-I-PDE}]/[\text{I-PDE}] = [T\alpha]/K_d$, is controlled by the concentration of transducin rather than its stoichiometry relative to that of native PDE.

For the affinity of the second inhibitor for PDEαβ-I, no estimate has been made, as its existence was unsuspected. However, Sitaramayya *et al.* (8) observed that in broken ROS light-activated PDE, which is transducin-activated PDE, needed much higher inhibitor concentration to be inactivated than does the trypsin-activated enzyme. In our hands (Figs. 2 and 3), activation by transducin *in vitro* produces mostly PDEαβ-I. The low affinity (0.4 μM) evaluated in ref. 8 from inactivation curves may then be taken as an order of magnitude guess for the affinity of I for PDEαβ-I in the equilibrium: $\text{I} + \text{PDE}\alpha\beta\text{-I} \leftrightharpoons \text{PDE}\alpha\beta\text{-I}_2$. With such an affinity, the rate constant for the dissociation of one inhibitory subunit from the native PDE-I_2 could be in the subsecond range.

Is the High Concentration of Transducin Found *in Vivo* Required for Full Activation of PDE? It would not make sense that, in the physiological response, light could not fully activate the PDE. The low rate of activation by transducin *in vitro*, as compared to proteolytic activation, is probably due to the low concentrations of active transducin that are used for *in vitro* assay, as compared to the concentration that may be reached *in vivo*, around a photoexcited rhodopsin molecule. In our assays, as in all other published experiments, the concentration of transducin is, at most, in the micromolar range. This appears sufficient to strip most PDE of one inhibitor, but not of both their inhibitory subunits. *In vivo*, within a few hundred milliseconds of the absorption of a photon, all of the transducin molecules surrounding the photoexcited rhodopsin are activated, and all of the Tα–GTP subunits are liberated. The local concentration of Tα–GTP in the cytoplasmic cleft between two discs may then reach 500 μM (19). This is 2 orders of magnitude higher than that commonly used *in vitro* and probably sufficient to fully activate, at least transiently, surrounding PDE molecules.

Exchange of Inhibitor Between the Three States of PDE Hinders the Estimate of the Specific Activity of PDEαβ-I. In Fig. 5, inhibitor exchange is observed in a sample in which the two original components, $\text{PDE}\alpha\beta\text{-I}_2$ and PDEαβ, had incubated together for a few minutes before the separation of the different complexes on the ion exchange column. No attempt has yet been made to reduce this time and study the exchange rate. The activity measurements on elution fractions (Figs. 2 and 4*B*) were always performed long after their isolation, so one expects that in each fraction an equilibrium has been reached. Therefore, the measured activity cannot be attributed to the single state that was originally isolated. The ratio of activities for fractions of the PDEαβ peak versus that of the PDEαβ-I peak is between 2 and 2.5. This value does not allow us to exclude one of two extreme hypotheses. The first would be that PDEαβ has exactly twice the activity of PDEαβ-I; the two catalytic subunits are functionally identical and work independently, each one being blocked by its inhibitor; removing one inhibitor releases exactly one-half of the total activity. The other hypothesis would be that the α and β subunits form a unique catalytic complex that is active only when both inhibitory subunits have been removed: only PDEαβ would be active, and the activity measured on the fractions from the PDEαβ-I peak would result entirely from the PDEαβ generated by exchange: $2\ \text{PDE}\alpha\beta\text{-I} \rightleftharpoons \text{PDE}\alpha\beta + \text{PDE}\alpha\beta\text{-I}_2$. In the first hypothesis, the PDE activity depends linearly on the inhibitor content of the sample, as if there were only one inhibitor per complex. The other, more interesting, alternative,which also applies for any intermediate case where PDEαβ-I has less than one-half the activity of PDEαβ, allows for nonlinear coupling between inhibitor removal and PDE activity and permits speculation on possible regulation mechanisms.

A Speculative Regulation Model. Dilution of transducin and exchange of inhibitor between inactive $\text{PDE}\alpha\beta\text{-I}_2$ and fully activated PDEαβ could regulate the overall PDE activity *in vivo*. The rapid termination of the rod physiological response requires that every step of the cGMP cascade be rapidly blocked after a flash (1). Photoexcited rhodopsin itself is rapidly inhibited by phosphorylation followed by arrestin binding (20). But for decoupling transducin from PDE, the GTPase rate of transducin seems too slow to account for a rapid turn-off correlated with the decay of Tα–GTP. We have already pointed out that *in vivo*, around a single photoexcited rhodopsin, the Tα–GTP concentration increases temporarily high enough to fully strip and maximally activate the PDE. But as Tα–GTP, which is very soluble, diffuses away into more distant membrane areas its concentration becomes too low to produce any more PDEαβ. The initially formed PDEαβ also diffuse away into areas where they encounter excess native $\text{PDE}\alpha\beta\text{-I}_2$. One might then speculate that exchange of inhibitor between $\text{PDE}\alpha\beta\text{-I}_2$ and PDEαβ could lead to a decrease of total PDE activity if PDEαβ-I has less than one-half the activity of PDEαβ. For this latter process to be kinetically significant, the dissociation rate constant of one subunit I from PDE-I_2 should be in the subsecond range, as discussed above. Both of these diffusion-dependent processes could allow a rapid quenching of the PDE activity that would precede the permanent inactivation of transducin resulting from GTP hydrolysis.

We thank Dr. Claude Pfister for many stimulating discussions and Dr. James Hurley for helpful comments on the manuscript.

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