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Activation of Retinal Rod Cyclic GMP-Phosphodiesterase by Transducin: Characterization of the Complex Formed by Phosphodiesterase Inhibitor and Transducin α-Subunit

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ABSTRACT **The GTP-binding subunit of transducin (Tα) activates the cGMP phosphodiesterase (PDE) of bovine retinal rods by relieving the constraint imposed by the inhibitory subunit PDEγ. We have isolated and characterized the complex Tα.GTPγS-PDEγ formed when Tα is activated by the nonhydrolyzable analog GTPγS. Sedimentation and light-scattering techniques demonstrate that, in contrast to free Tα.GTPγS, which is soluble, the Tα.GTPγS-PDEγ complex, as well as Tα.GTP-PDEγ, is membrane bound at cytosolic ionic strength. It is eluted from the membrane at low ionic strength as a monomeric and 1:1 stoichiometric complex. The relative affinities of PDEγ for PDEαβ and for Tα.GTP are discussed.**

Key words: G-protein, phototransduction

INTRODUCTION

In retinal rod outer segments (ROS)*, illumination triggers a cascade of reactions initiated by photoexcited rhodopsin and mediated by the GTP-dependent protein transducin (T), which results in a fast and intense hydrolysis of cGMP by a specific phosphodiesterase (PDE). This cascade is an archetype of the ubiquitous G protein-mediated transduction system [1–4] between membrane receptors (to hormones, neurotransmitters, etc.) and cytoplasmic effectors of various types: cyclase, phosphodiesterase, phosphatidylinositol-specific phosphodiesterases, etc.). In ROS, various biophysical as well as biochemical methods have allowed precise determinations of the kinetics and stoichiometry of the successive steps of the cascade. First, one photoexcited rhodopsin catalyzes the GDP/GTP exchange reaction on the nucleotide-binding subunit Tα of many transducin molecules by sequential collision coupling.[5–7] The newly formed Tα.GTP molecules dissociate from the membrane-associated Tβγ and become soluble at cytoplasm ionic strength.[8,9] Then in a second step, each "active" Tα.GTP activates in turn a membrane-bound PDE molecule by relieving the constraint imposed by a 13-kDa inhibitory subunit (PDEγ) on the two large catalytic subunits PDEαβ.[10]

The mechanism through which the inhibition is relieved remained uncertain, as it was not clear whether it involves the physical dissociation of PDEγ from PDEαβ and eventually from the membrane. It has been proposed that, in isotonic medium conditions, the PDE inhibitor would become soluble together with Tα.GTP[11], but the Tα.GTP-PDEγ complex has not been isolated and its solubility or membrane attachment has not been characterized. On the contrary, Wensel and Stryer[13] demonstrate that PDEγ stays on the membrane when PDE is activated. Besides, from kinetic studies on light and trypsin-activated PDE, it has been suggested [12] that Tα remains bound to PDEαβ.

In the present study, we demonstrate the existence of the Tα.GTPγS-PDEγ complex. It can be isolated as a 1:1 stoichiometric complex and separated from PDEαβ and from free Tα.GTPγS by ion-exchange chromatography. This complex is membrane bound in isotonic medium and can be extracted from the membrane, as well as from the catalytic units of the PDE, only by low-ionic-strength washing. Further study by the near-infrared light-scattering technique confirms that at cytosolic ionic strength the interaction of Tα.GTP with the cGMP phosphodiesterase induces the binding of the soluble Tα.GTP to the membrane.

MATERIALS AND METHODS

Sample Preparation

ROS membranes were prepared as in reference 8. The thawed pellets were homogenized, illuminated, and then washed in medium salt buffer, eliminating some minor proteins not concerned in this study. Extraction and "crude" purification procedures were then carried out, using the light, nucleotide, and ionic-strength dependence of binding of the species.[8] The different steps are summarized at the top of Figure 1.

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*Abbreviations: ROS, rod outer segments; GTPγS, guanosine 5′-(3-0-thio)triphosphate; PDE, cGMP-phosphodiesterase; PDEαβ, PDEγ, its αβ- or γ-subunits; T, transducin; Tα.GTP or Tα.GTPγS, its α-subunit with the bound nucleotide; Tβγ, its βγ-subunit; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethylsulphonylfluoride; SDS, sodium dodecylsulfate.

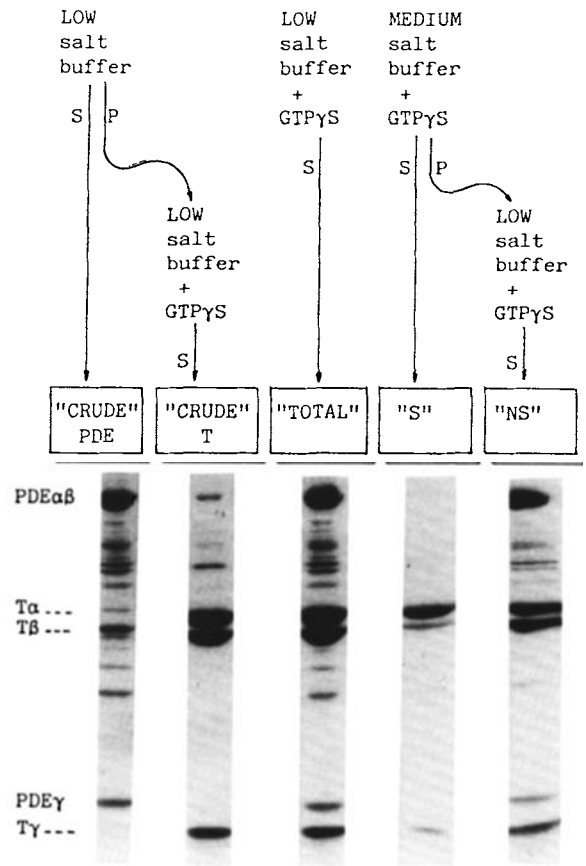

Fig. 1. Preparation procedures and polypeptide pattern of ROS extracts. Illuminated and washed ROS suspensions (see Methods) were subjected to the indicated sequences of extractions. The supernatants (S) were analyzed on SDS gels. P = pellet. "Crude" PDE contains some $T\beta\gamma$ as contaminant; "S" contains essentially $T\alpha$, with traces of $T\beta\gamma$, but no $PDE\gamma$.

For instance, the supernatant of a suspension in low salt buffer without nucleotides yields crude PDE, and a subsequent extraction of the pellet in low salt buffer supplemented with GTPγS allows to recover crude T in the supernatant. Samples "S" and "NS" are supernatants from two successive extractions: the first, in medium salt buffer with added GTPγS, contains essentially $T\alpha$, contaminated by traces of $T\beta\gamma$, and no $PDE\gamma$ is detected; the second, in low salt buffer with added GTPγS, contains the remaining $T\alpha$ and contains also all the $T\beta\gamma$ and all the PDE, in particular all the $PDE\gamma$. Low salt buffer (in mM): Hepes 5, DTT 1, pH 7.5. Medium salt buffer: HEPES 20, KCl 120, DTT 1, pH 7.5. Rhodopsin concentration in all extracts was 2 mg/ml. GTPγS was used at 100 μM.

Protein Chromatography

Separation and purification of proteins on ion exchange on a Pharmacia FPLC Polyanion SI column[14] was performed with a Na_2SO_4 gradient (from zero to 300 mM) in a buffer containing (in mM): HEPES 20 pH 7.5, $MgSO_4$ 10, phenylmethylsulfonylfluoride (PMSF) 0.1, 2-mercaptoethanol 5. Gel filtration was carried out on a Pharmacia Superose-12 column in a buffer containing (in mM) HEPES 20 pH 5.5, NaCl 120, $MgCl_2$ 2, PMSF 0.1, 2-mercaptoethanol 5.

The protein content of "crude" extracts or of purified solutions was estimated through the Coomassie Blue colorimetric technique with BSA as a standard. SDS polyacrylamide gel electrophoresis (SDS PAGE)[15] was performed on 10–15% acrylamide gradient gels. Gels were stained with Coomassie Blue, and estimates of the relative protein content were derived from densitometry of the gels.

PDE Assays

The PDE activity was measured by the pH-metric method[16], at 20°C, in a final volume of 2 ml of a medium containing (in mM) HEPES 10 pH 7.5, $MgCl_2$ 2, KCl 120, supplemented with either dark-adapted ROS membranes (1.25 μM rhodopsin final concentration) or crude PDE (6 nM final). The reaction was initiated by addition of $T\alpha$.GTPγS or $T\alpha$.$PDE\gamma$ (75 nM final) along with cGMP (0.4 mM final). In the presence of $T\alpha$.GTPγS, the PDE activity was $7.5 \times 10^{-8} H^+$/min.

Light-Scattering Measurements

Near-infrared light-scattering measurements were performed as described in reference 17. Briefly, the changes in transmitted light intensity were recorded at 900 nm after a flash of 0.5-msec duration that bleached 0.4% of the rhodopsin. The ROS suspension (3.8 μM rhodopsin final concentration) was gently homogenized by passing it several times through a syringe needle (22 gauge) that had been sharply bent. Gentle homogenization of ROS is important to avoid the rise of strong and complex additional light-scattering changes that are observed in more rigorously homogenized or osmotically shocked membrane preparations. These changes are also PDE- and light-dependent and are thought to reflect disk-vesicle aggregation.[18] The suspension medium was (in mM) KCl 150, HEPES 10 pH 7.0, $MgCl_2$ 1, DTT 1, GTP 0.1. The samples were preincubated for 10 min before the flash. Experiments were performed at 20°C.

RESULTS

Identification of the $T\alpha$.GTPγS-$PDE\gamma$ Complex

A permanent activation of transducin and phosphodiesterase is induced by illuminating ROS membranes in the presence of GTPγS. The products of the T-PDE interaction can be extracted (Fig. 1) in one step at low ionic strength ("total" extract) or sorted into two classes: those soluble under cytosolic conditions (extract "S" in medium salt buffer, which contains mainly $T\alpha$, and no $PDE\gamma$) and those soluble only in low-ionic-strength buffer (extract "NS," which contains $T\beta\gamma$, $PDE\alpha\beta$, $PDE\gamma$ and some $T\alpha$; see Methods).

The ion-exchange chromatography elution profile of a "total" extract (Fig. 2C) displays the expected major

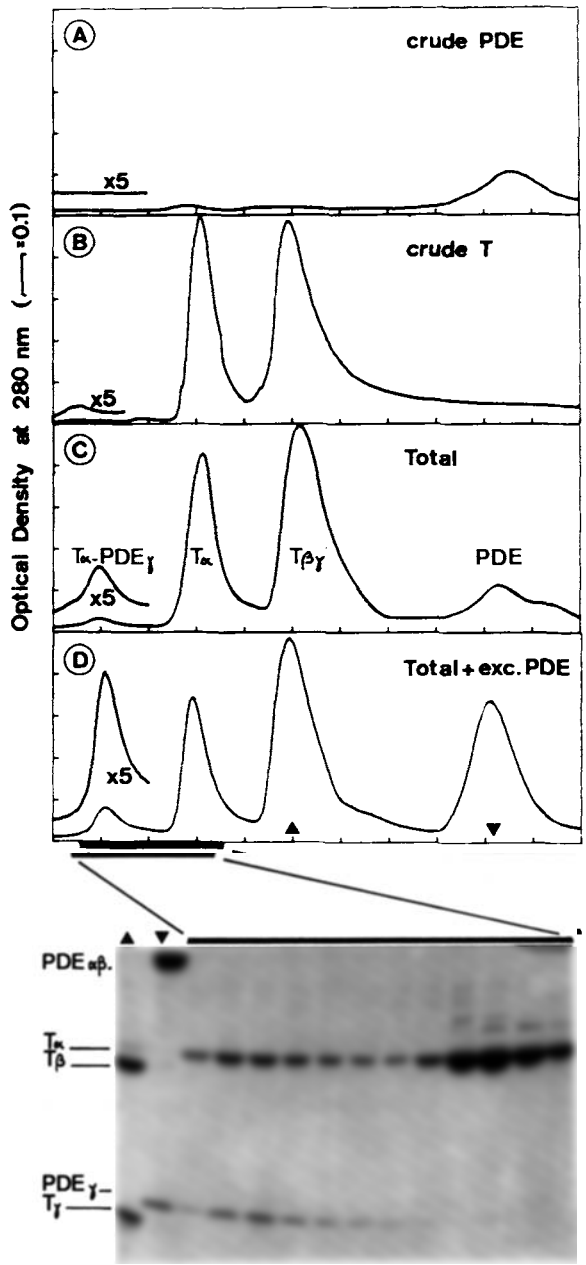

Fig. 2. Elution profiles from anion-exchange chromatography of ROS extracts. Each extract (A–D) was obtained from ROS containing 10 mg rhodopsin. In D, ROS were supplemented with excess PDE (see legend of Fig. 4). SDS-polyacrylamide gel electrophoresis (PAGE) analysis of fractions from D reveals the presence of the Tα.GTPγS-PDEγ complex in the first peak (eluted at 65mM Na_2SO_4). An expanded (x 5) optical density scale is shown for the first peak.

peaks of Tα.GTPγS, Tβγ and PDEαβγ, also present in the elution profiles of crude T (Fig. 2B) and crude PDE (Fig. 2A). One notices, however, a small additional peak revealed by SDS gel to contain both Tα.GTPγS and PDEγ. Densitometry of stained gels of the eluted fractions demonstrates an exact superposition of the elution profiles of these two polypeptides (not shown). Since neither Tα.GTPγS in crude T (Fig. 2B) nor PDEγ in "crude PDE" (Fig. 2A) eluted at the position of this new peak, we can exclude the possibility of these two species fortuitously coeluting as independent, unassociated polypeptides. Additional lines of evidence support the conclusion that this peak represents the complex Tα.GTPγS-PDEγ: a relative increase in amplitude of this "complex" peak is observed when an excess of crude PDE is added to the ROS preparation before "total" extraction (Fig. 2D); both polypeptides Tα and PDEγ are enhanced in the "complex" peak, indicating that the excess PDE draws on the pool of Tα.GTPγS.

It is generally assumed that PDE activation by Tα can be obtained only in the presence of membranes. Yet, we observed that Tα.GTPγS can partially activate PDE in the absence of membranes (20% of the activation obtained in the presence of membranes) in accordance with Fung and Nash.[19] Interestingly, we also observed the presence of a "complex" peak in the elution profiles of a mixture of purified PDE and purified Tα.GTPγS solutions, incubated in the absence of ROS membranes. This is consistent with the notion that the formation of this complex is correlated with the activation mechanism of PDE.

Furthermore, we checked that the Tα.GTPγS engaged in the complex was unable to activate PDE. Two samples containing the same amount of Tα.GTPγS, one coming from the Tα.GTPγS-PDEγ peak, the other from purified Tα.GTPγS, were assayed for their ability to activate the PDE of dark-adapted ROS membranes (see Methods). We found that the PDE activity obtained upon addition of Tα.GTPγS-PDEγ was 30 times lower than that obtained upon addition of free Tα.GTPγS.

To estimate the size of the complex, a mixture of crude PDE and crude T solutions was eluted on a gel filtration column. Analysis of the gel of eluted fractions shows that a peak containing all of the Tα.GTPγS and a fraction of the PDEγ pool emerged at an elution volume corresponding to a molecular weight of 40 kDa, according to the calibration obtained with soluble proteins (Fig. 3). Elution of pure Tα.GTPγS gave a peak at the same position. The complex, which is necessarily larger than Tα.GTPγS alone, is therefore slightly retarded on this column. A similar retard was observed for Tβγ and PDEαβ, both of which are membrane-associated proteins. The elution volume of the Tα.GTPγS-PDEγ complex indicates that there is no more than one Tα per complex (Fig. 3), whereas the slight shift observed with respect to the expected elution volume suggests that the complex is membrane bound, as discussed and confirmed below.

The stoichiometry of the complex was also estimated by densitometry of the stained SDS gel of the ion-exchange column fractions. A ratio of 3 to 4 was consistently measured for the staining of the two

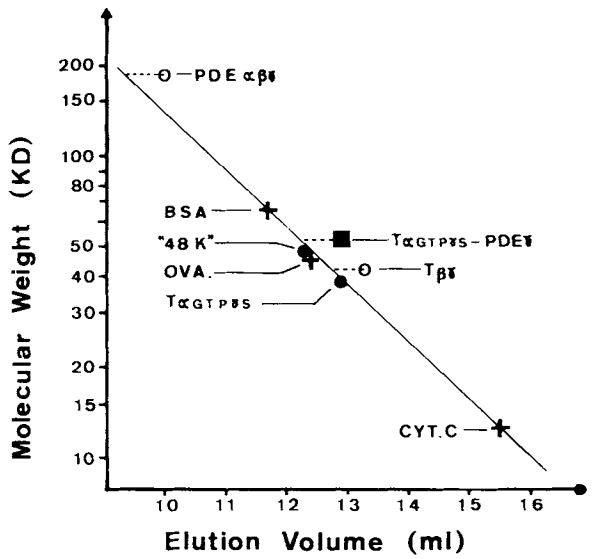

Fig. 3. Gel filtration of the Tα.GTPγS-PDEγ complex. +, calibration with soluble proteins (bovine serum albumin, ovalbumin, cytochrome C). Soluble ROS proteins of known molecular weights, "48 K" and Tα.GTPγS (●), are eluted acccording to the above calibration, but the elution volumes for the two nonsoluble ROS proteins PDE and Tβγ (○) are weakly but systematically increased with respect to what would be expected for a soluble protein of the same molecular weight. The elution volume observed for the complex leads us to propose a 1:1 structure (■, Mw = 50 kD), which corresponds to a derivation from calibration of the same order as for Tβγ and PDE. The 2:1 stoichiometry (Mw = 90kD) would deviate much more from calibration.

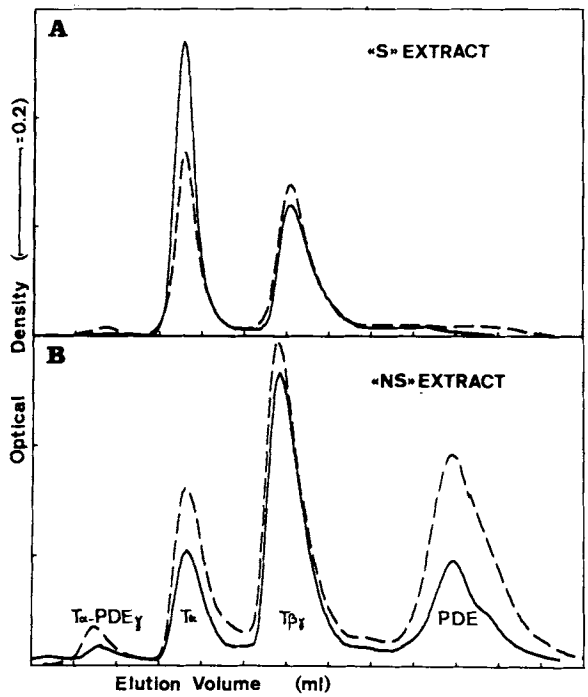

Fig. 4. Anion exchange chromatography of "S" extracts (part A) and "NS" extracts (part B) of 20-mg rhodopsin ROS aliquots supplemented (dotted lines) or not (continuous lines) with excess PDE. In the supplemented aliquots, the membrane-bound PDE pool has been increased by a factor of 3–4 by addition of crude PDE to the membrane suspension before extraction. The crude PDE contains some Tβγ as a contaminant (see Fig. 1).

component polypeptides. This reflects the molecular weight ratio of Tα vs. PDEγ. We therefore propose that the complex has a 1:1 stoichiometry.

Membrane Attachment of the Tα.GTPγS-PDEγ Complex

Whereas PDE and Tα.GDP-Tβγ are perimembraneous proteins that can be separated from ROS membranes only in low-ionic-strength buffer, the active Tα.GTP or Tα.GTPγS species are soluble, i.e., largely dissociated from the membrane in isotonic medium.[9] To evaluate the solubility of the Tα.GTPγS-PDEγ complex, we measured its extractibility in two successive elutions from ROS membranes (extract "S" and extract "NS," see Fig. 1). Whereas the greatest part of Tα is found in the first extract (Fig. 4A), PDE and the Tα.GTPγS-PDE complex are mainly detected in the second extract (Fig. 4B). The solubility of these proteins was compared (Table I) with that of the very soluble "48 kDa" protein.[20] The Tα.GTPγS-PDEγ complex is found to be membrane bound like PDE rather than soluble like Tα.GTPγS. Hence the association with PDEγ implies for Tα.GTPγS a binding to the membrane. This was further documented by comparing the "S" and "NS" extracts of ROS aliquots which had been supplemented or not with excess PDE prior to extraction (Fig. 4). While the presence of excess PDE does not influence the yield of Tβγ extraction at medium ionic strength, it significantly reduces the amount of soluble Tα.GTPγS released (Fig. 4A). On the other hand, it enhances the quantity of membrane-associated Tα.GTPγS extractable at low ionic strength (Fig. 4B).

Infrared Light-Scattering Measurements Confirms the Membrane Attachment of the Tα.GTP-PDEγ Complex

Near-infrared light-scattering technique can be used to monitor the light-triggered transducin reactions[9,17] on a much faster time scale, therefore permitting the use of GTP instead of GTPγS. A "dissociation" signal observed upon flash illumination in the presence of GTP has been interpreted[9,21] as due to the actual release of solubilized Tα.GTP from the ROS. The amplitude of the signal related to with the amount of released Tα. The above observed membrane attachment of the Tα.GTP-PDEγ complex implies now that only the excess Tα.GTP, not interacting with PDE, is released and gives rise to the signal. In situ transducin is present in about 5-fold excess with respect to PDE.[12] Most (80%) of the Tα.GTP will therefore be released upon a flash of saturating light intensity,

TABLE I. Solubility of the Complex Tα.GTPγS-PDEγ Compared to That of Other ROS Proteins*

	% Yield in extract "S"/ Yield in "S" + "NS"	Normalized solubility
"48-kDa" protein	0.95	1
Tα.GTPγS	0.70	0.7
Tβγ	0.35	0.4
PDEαβγ	0.10	0.1
Tα.GTPγS-PDEγ	0.10	0.1

*Sequential extracts "S" and "NS" were chromatographed (as in Fig. 4) and the protein contents of the eluted fractions were assayed by densitometry of stained gels. Extraction of "48-kDa" protein was performed in the dark without nucleotide.

thus giving rise to a large dissociation signal. We observed (Fig. 5A) that additions of excess PDE to ROS lead to significant reductions of this dissociation signal. This reduction was linear with the amount of added PDE (Fig. 5B). Estimates of the Tα and PDE amounts present in these assays lead us to deduce a nearly quantitative binding of Tα.GTP to PDE, on a mole to mole basis: 1 mole Tα.GTP became membrane-bound when about 2 moles PDE was added (see legend for Fig. 5).

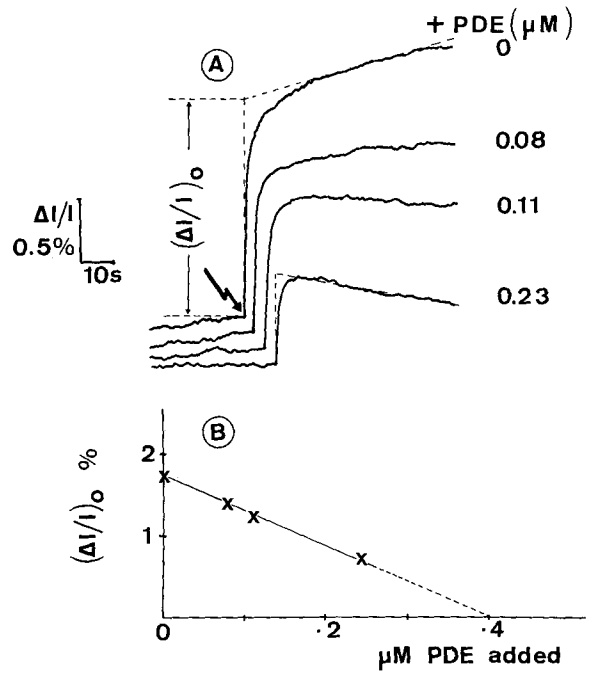

Fig. 5. Reduction of the light-scattering "dissociation signal"[17] in ROS membrane suspensions by addition of excess PDE. A. Various amounts of "crude PDE" were added to a ROS suspension (3.8 μM rhodopsin) at final concentrations indicated, and the mixture was incubated for 10 min before the flash. The inital amplitude of the signal $(\Delta I/I)_0$ was determined from extrapolation of the trace to t = 0. B. Plot of $(\Delta I/I)_0$ vs. the amount of added PDE. By extrapolation, the signal would vanish, and all the Tα would be membrane-associated, at 0.4 μM PDE added. The total amount of Tα present in this ROS suspension was estimated from the saturation of the "binding signal"[17] to be 5% of rhodopsin, i.e., 0.19 μM. Control experiments revealed that the "binding signal" was not significantly modified by addition of PDE. Taking into account the PDE present in the ROS suspension (1–2% of rhodopsin,[12] i.e., 0.04–0.08 μM) 0.19 μM Tα would be membrane bound in the presence of 0.44–0.48 μM PDE.

DISCUSSION

We present here evidence that the activation of PDE by transducin proceeds through the formation of a membrane-bound complex, which can be extracted from ROS membrane only by low-ionic-strength washing. After solubilization, the binary complex Tα.GTPγS-PDEγ appears to be dissociated from the catalytic units PDEαβ from which it can be separated by ion exchange as well as by molecular sieve chromatography. Unlike Yamazaki et al,[11] we observe that PDE activation in ROS implies a binding of Tα.GTPγS to the membrane rather than the release of PDEγ from the membrane. The difference might be due to their use of frog rather than bovine ROS. Our results rule out the formation of Tα.PDEαβ as inferred by Sitaramayya et al.[12]

From a gel analysis of the polypeptide content of the various peaks in the "total" extract elution profile, one may get an evaluation of the equilibrium constant A = k1/k2 of the exchange reaction in solution:

$$T\alpha.GTP\gamma S + PDE\alpha\beta\text{-}PDE\gamma \underset{k2}{\overset{k1}{\rightleftharpoons}} T\alpha.GTP\gamma S\text{-}PDE\gamma + PDE\alpha\beta$$

The evaluation is based on the assumption of a 1:1 stoichiometry of association of PDEγ with PDEαβ in native ROS, as suggested by Wensel and Stryer.[13] It requires only the estimates of the relative amounts of Tα.GTPγS in the first vs. the second peak and that of PDEγ in the first vs. the fourth peak of figure 2C or D. One gets A = 1/15, but preliminary results show that this ratio increases when the Mg^{++} concentration in the medium decreases. This value seems to indicate a low efficiency for the activation of PDE by transducin. In fact, a weak but clear activation of PDE by permanently activated Tα has been detected either in the absence of membranes (present report and reference 19) or in highly diluted ROS suspensions (rhodopsin concentration 50 nM[13]). By contrast, in conditions closer to the in situ situation, when both T and PDE are initially membrane associated, we found that the addition of excess PDE strongly reduces the amount of Tα.GTP or Tα.GTPγS released in the solution, as shown by two different techniques (Figs. 4,5). This can only result from an interaction of an increased fraction of the total activated Tα with the excess PDE bound onto the membrane. The evaluation, from light-scattering experiments, of the stoichiometry of bound Tα.GTP vs. added PDE (Fig. 5) is only approximate and must be taken with caution, but it is clear that in these assay conditions (isotonic ionic strength, more concentrated (4 μM) and native

ROS membranes), the binding equilibrium is shifted far more in favor of the complex Tα.GTP-PDEγ than in solution or in dilute ROS suspensions.

The fact that in solution only the binary complex Tα.GTPγS-PDEγ is observed, and not a quarternary complex PDEαβγ-Tα.GTPγS, suggests, but does not prove, that in situ the membrane-bound Tα.GTPγS-PDEγ complex is also dissociated from the catalytic units of the PDE, or at most only weakly attached to them. This would imply that the binding of PDEγ to PDEαβ or to Tα.GTPγS is mutually exclusive.

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