Biochemistry

 Volume 33, Number 51 December 27, 1994

Accelerated Publications

Modulation of the GTPase Activity of Transducin. Kinetic Studies of Reconstituted Systems†

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Received October 3, 1994; Revised Manuscript Received October 31, 1994®

ABSTRACT: We seek to define the influence of retinal cGMP phosphodiesterase (PDE) on the GTPase activity of transducin (T). A novel stopped-flow/fast filtration apparatus [Antonny, B., et al. (1993) *Biochemistry 32*, 8646−8653] is used to deliver TαGTP free of rod outer segment (ROS) membranes to a suspension of phospholipid vesicles bearing holoPDE. As measured by a pH electrode, the decay of cGMP hydrolysis from these samples, which contain no other proteins but Tα and holoPDE, requires GTP hydrolysis and occurs in 40 s. The addition of Tβγ to the vesicles does not accelerate this deactivation. When ROS membranes are urea-stripped, reconstituted with transducin + holoPDE, and illuminated, the injection of an amount of GTP that is substoichiometric to holoPDE gives a cGMP hydrolysis pulse that lasts for 30 s. However, the same reconstitution performed with ROS stripped by extensive dilution in isotonic buffer results in a deactivation time of only 8 s, which resembles the 7 s observed with native ROSs. With these isotonically stripped ROSs, when GTP injection comes after a first injection with GTPγS, the cGMP hydrolysis pulse is lengthened and lasts for 17 s; with urea-washed ROS, no such lengthening is observed. These results clearly demonstrate that holoPDE by itself cannot enhance the GTPase activity of transducin, even when the two proteins are localized on a membrane surface. Instead, they point to the existence of a membrane-bound, urea-sensitive protein factor that activates the GTPase of Tα in the transducin−holoPDE complex.

In signaling cascades mediated by heterotrimeric G-proteins, termination of the response ultimately implies GTP hydrolysis by the Gα-subunits (Bourne et al., 1991). In vertebrate visual transduction, the GTPase activity of transducin, besides being absolutely required, must also occur swiftly within a second or so (Hurley, 1994). Measuring this activity has been a major technical challenge, partly because unlike those of other G-proteins, the α-subunit of transducin cannot be induced to spontaneously take up GTP (Higashijima et al., 1987). Photoexcited rhodopsin (R*) must be present to catalyze the GDP/GTP exchange on Tα. Upon dissociation from R* and Tβγ, TαGTP noncatalytically activates a multimeric effector, the cGMP phosphodiesterase, PDEαβ(γ)$_2$. Up to two molecules of TαGTP can bind to a holoPDE tetramer, where they displace the PDEγ-subunits from inhibitory sites on the catalytic PDEαβ moieties. Reinhibition of PDEαβ requires GTP hydrolysis by Tα and restitution of PDEγ to its inhibitory site.

To measure the GTPase activity of Tα, several methods are available, all requiring the catalyzing power of R*. In the steady state method (Pagès et al., 1992), a large amount of GTP is added to illuminated ROS membranes; the GDP

† This work was supported by a grant from the Human Frontier Science Program Organization.

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® Abstract published in *Advance ACS Abstracts,* December 1, 1994.

0006-2960/94/0433-15215$04.50/0

produced is then assayed at several time points. The difficulty with this technique is that it measures other times in addition to the GTP hydrolysis time since the cascade is allowed many turnovers. This is nicely avoided in the direct single-turnover method (Arshavsky & Bownds, 1992). ROS membranes are illuminated so that transducin binds to R* and Tα releases its GDP. The reaction is started by adding an amount of [γ-P^{32}]GTP that is substoichiometric to the Tα present, and the exponential production of radioactive phosphate is sampled at several time points. In the indirect single-turnover method, a similar experimental protocol is followed, except that one now time-resolves the kinetics of cGMP hydrolysis with a pH electrode (Angleson & Wensel, 1993). GTP hydrolysis by Tα is thus monitored indirectly via the GTPase-dependent deactivation of holoPDE.

A common feature of both techniques is that the ROS membranes needed to activate transducin remain in the reaction vessel where GTP hydrolysis occurs. If there were factors in the disk membrane that regulate transducin GTPase, the interpretation of some results might be ambiguous. To circumvent this difficulty, we devised a novel technique of stopped-flow/fast filtration where photoexcited ROS membranes are retained on a 0.22-μm filter, from which soluble TαGTP can be eluted with a flow of buffer containing GTP or GTPγS (Antonny et al., 1993). The thus purified TαGTP is collected in a cuvette, and the time it takes to switch to the GDP conformation is measured by following its tryptophan fluorescence. The activation and deactivation of transducin are thus physically separated, and its GTPase activity can be measured in the most minimal of environments. But given the optical nature of the measurement, the addition of proteins, which might be very fluorescent, or of membranes, which scatter strongly, has not been feasible.

Lastly, time-resolved microcalorimetry was used to follow the time course of cGMP hydrolysis from native ROS under conditions that approximate those *in vivo*, namely, high membrane and GTP concentrations and very low illumination (Vuong & Chabre, 1991). In these native ROSs, GTP hydrolysis is strictly required for terminating the cascade, occurs in about 1 s, and is thus kinetically compatible with the rod cell's photoresponse. Yet, as measured by the stopped-flow/fast filtration method, soluble TαGTP in the absence of ROS membranes hydrolyzes its GTP and switches to the GDP form in 20 s (Antonny et al., 1993). This very clear-cut dichotomy in kinetics naturally raises the following question: During the normal course of events in the native ROS, what speeds up the slow, intrinsic GTPase activity of Tα? The obvious candidate is PDEγ, which is the most immediate partner of Tα in the active transducin−PDE complex. Moreover, in solution the affinity of PDEγ for TαGTPγS is ~0.1 nM (Otto-Bruc et al., 1993), and soluble TαGTP−PDEγ can be obtained by simply having excess PDEγ in the cuvette that receives TαGTP from the stopped-flow/fast filtration apparatus. In solution, the transition from TαGTP−PDEγ to TαGDP−PDEγ occurs in 24 s (Antonny et al., 1993), indicating that PDEγ has no effect on the GTPase of isolated Tα. But using the technique of direct single turnover, Arshavsky and Bownds (1992) reported that when PDEγ or holoPDE is added to the ROS membrane preparation, the GTPase activity of Tα is increased severalfold. On the other hand, using both direct and indirect single-turnover methods, Angleson and Wensel (1993) indicated that PDEγ is not a GTPase-activating protein (GAP) for Tα; it is really an as yet unidentified membrane factor that fulfills this role.

In view of these somewhat conflicting conclusions, a simpler approach to reconstitution would be useful. Indeed, if active PDE consisting of holoPDE and TαGTP is reconstituted on phospholipid vesicles that contain no other proteins, at what rate will it deactivate? Will this rate resemble that of soluble TαGTP, as reported by Antonny et al. (1993), or will it be more reminiscent of native ROS, as reported by Vuong and Chabre (1991)? An unambiguous answer will help resolve two important issues: (i) Does any part of holoPDE, be it PDEγ and/or PDE$\alpha\beta$, have any effect on the GTPase of the associated Tα? (ii) What influence does membrane localization have on this GTPase activity?

We have constructed a new version of the stopped-flow/fast filtration apparatus (Antonny et al., 1993), where the TαGTP stream is collected in a special pH measurement cuvette containing phospholipid vesicles on which holoPDE is reconstituted. Once activated by the incoming TαGTP, cGMP hydrolysis by this membrane-associated holoPDE is monitored with a pH electrode. Termination of cGMP hydrolysis in such preparations absolutely requires GTP hydrolysis and takes place within 40 s (1/e time). This result unequivocally confirms our earlier finding that PDEγ is not capable of accelerating the GTPase of Tα, extends this disqualification to PDE$\alpha\beta$, and shows that membrane localization in itself plays no role in how fast TαGTP hydrolyzes its GTP. As such, our results agree with those of Angleson and Wensel (1993, 1994) and lend credence to their proposal that a membrane protein present in ROS, which is distinct from holoPDE, is the GAP. On the other hand, our findings directly contradict the report by Pagès et al. (1992, 1993) that holoPDE, and in particular PDE$\alpha\beta$, is the GAP. They are also in disagreement with the assertion by Arshavsky and Bownds (1992) and Arshavsky et al. (1994) that PDEγ is the GAP. We then applied the strategy of indirect single turnover to three types of ROS preparations: native ROS and ROS stripped of transducin and PDE by extensive dilution in isotonic buffer or by washing with 4 M urea. The stripped ROSs are reconstituted with transducin and holoPDE. The PDE deactivation times (1/e) are, on the one hand, 7 ± 1 and 8 ± 1 s for native and isotonically stripped ROSs, respectively, and, on the other hand, 28 ± 2 s for urea-washed ROSs. The very slow deactivation time obtained with urea-washed ROSs resembles that of soluble TαGTP (Antonny et al., 1993), while with native and isotonically stripped ROSs, this time is remininscent of the fast deactivation kinetics observed via time-resolved microcalorimetry (Vuong & Chabre, 1991). It seems clear that the GTPase of Tα in the active transducin−PDE complex is enhanced by a urea-sensitive, tightly membrane-bound protein cofactor.

EXPERIMENTAL PROCEDURES

Stopped-Flow/Fast Filtration Apparatus. This device is modified from the version described in Antonny et al. (1993) to accommodate fast pH measurements (Figure 1). Illuminated bovine ROSs (200 μg of rhodopsin) are placed on a 0.22-μm syringe filter (Millex GV, 25-mm diameter, Millipore S.A., Molsheim, France), which reliably retains the ROS fragments but allows any solubilized proteins to

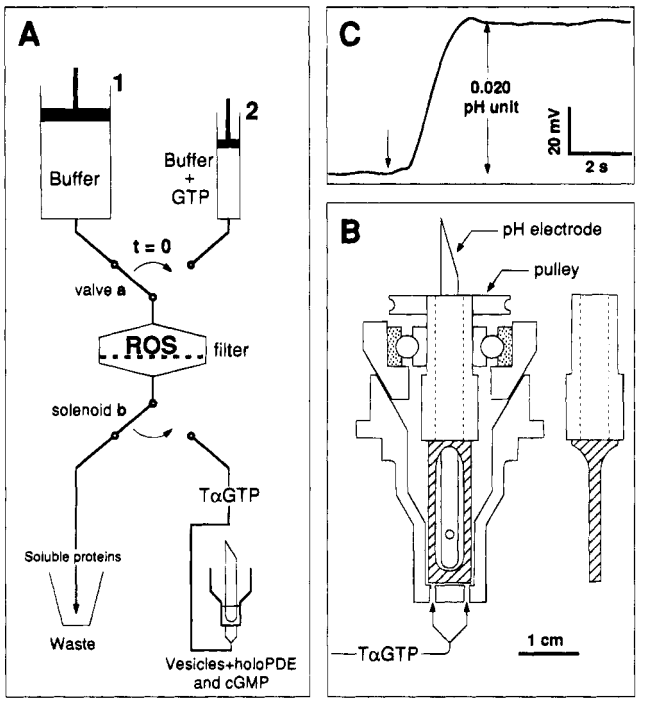

FIGURE 1: (A) Principle of operation. Syringes 1 and 2 contain 30 mL of buffer and 1.1 mL of buffer + 50 μM GTP or GTPγS, respectively. At the start of the experiment, pneumatically actuated valve a connects syringe 1 to the Millex filter inlet to permit a 15-s wash of the deposited ROS (200 μg of rhodopsin). During this wash, the eluate is sent to the waste. At zero time, valve a is actuated to connect syringe 2 to the Millex filter inlet. After a delay of ca. 0.3 s, solenoid b is switched to send the TαGTP or TαGTPγS stream into the pH measurement cuvette. (B) pH measurement cuvette; it is made out of polished Plexiglas and consists of a cylindrical part and a conical expansion chamber (see text). The Tα stream enters via two injection ports at the bottom of the cylindrical part. The initial 500 μL of solution, which contains vesicles + holoPDE and cGMP, resides in the cylindrical part and is just enough to reliably wet the pH electrode diaphragm. The Delrin stirrer (front and side views) is a tube whose lower part is machined to a flat, hollow blade (hatched area). Its top, cylindrical part is fitted with a ball bearing and a pulley. The pH electrode reaches into the cuvette via the central hole of the Delrin stirrer. (C) Response time of the instrument. 700 μL of measurement buffer + 200 μM HCl were injected into 500 μL of measurement buffer already resident in the cuvette. The arrow marks the time of actuation of valve a.

pass through. At its inlet, the filter can be connected via a 2-way, pneumatically actuated valve (a) to syringe 1, which contains just buffer (30 mL), or to syringe 2, which contains the same buffer (1.1 mL) supplemented with GTP or GTPγS. Syringes 1 and 2 are pressurized at 4.5 bar with regulated compressed air. The filter's outlet either is diverted into the waste or feeds into a special pH measurement cuvette. The 2-way solenoid outlet valve (b) is triggered by an optical proximity switch, which detects a reflector mounted on the piston of syringe 2. In a typical experiment, the Millex filter first receives a 15-s wash with buffer alone from syringe 1, which removes all soluble proteins from the ROS but does not affect transducin, as it is tightly bound to R* in the absence of nucleotides. At zero time, the inlet valve a is switched to syringe 2 to start the flow of buffer + nucleotide, which elutes activated Tα from the filter-retained ROS. After a suitable delay of ca. 0.3 s, the proximity switch flips the outlet valve b to direct the flow toward the cuvette, which contains a suspension of phospholipid vesicles bearing holoPDE. This delay allows the dead volume downstream from the filter to be sent to the waste.

The cuvette is designed to optimize mixing between the injected Tα stream and the resident vesicle suspension. The incoming stream enters the cuvette via two ports to minimize the formation of a bolus that is different in density from the surrounding vesicle suspension. Vigorous and rapid mixing is accomplished with a custom-built stirrer (Figure 1). This is a Delrin tube whose lower part is machined to a flat, hollow blade. The cylindrical upper part of the tube is fitted with a ball bearing and a pulley so that it can be belt-driven by a small electric motor. The tip of the stirrer's blade is positioned ~0.1 mm above the two injection ports, so that the Tα stream entering the cuvette is swiftly drawn into the turbulent zone set up by the rotating blade. The pH electrode (3-mm diameter, Model U402-M3-S7/60, Ingold Messtechnik GmbH, Steinbach/Ts., Germany) traverses the central hole of the Delrin tube to descend into the cuvette. The polished Plexiglas cuvette consists of a cylindrical part and a conical part. The former is made as narrow as is allowed by the orbit of the stirrer's blade, so that the small, preinjection volume of vesicle suspension can reach the diaphragm of the pH electrode. The conical part is an expansion chamber: Once the injection of Tα is over, the final volume of liquid is not so spread out that sampling by the pH electrode becomes too localized, as would be the case if the cuvette were only a tall, thin cylinder. Before each series of experiments, the Plexiglas cuvette, Delrin stirrer, and pH electrode were passivated with a BSA solution (1 mg/mL) for 30 min. The stopped-flow/fast filtration apparatus and the cuvette are thermostated at 25 °C with a circulating water bath.

The volume of vesicle suspension in the cuvette before Tα injection is 500 μL. The volume of injected Tα is 700 μL, the volume required to fully elute Tα from the filter-retained ROS (Antonny et al., 1993). At 4.5 bar, the flow rate through the Millex filter is such that this injection takes about 1 s. The instrument's response was assessed by injecting 700 μL of buffer containing 200 μM HCl into 500 μL of buffer in the cuvette (Figure 1). The plateau is reached within ~2 s, which is a combination of the injection time of 1 s, the electrode's response of 0.7 s, and the mixing between the resident and injected volumes. The pH electrode output was amplified 50-fold with a high-input impedance operational amplifier (Model AD-549LH, Analog Devices, Norwood, MA), filtered with a 0.22-s RC circuit, and digitized at a rate of 8.5 samples/s. The raw data were differentiated using the Savitsky−Golay (1964) algorithm (5- or 7-point) to obtain the rate of cGMP hydrolysis, which is directly proportional to the concentration of active PDE since the cGMP levels used were well above the 70 μM K_m of the enzyme.

Buffers. Isotonic buffer contains the following (mM): KCl (120), $MgCl_2$ (1), Hepes (20, pH 7.5). Hypotonic buffer contains the following (mM): $MgCl_2$ (0.1), Hepes (5, pH 7.5). For experiments with phospholipid vesicles, the measurement buffer was the same as the isotonic buffer except it contained 10 mM Hepes instead of 20. For the classical pH metric experiments, isotonic buffer was used.

Extraction of Transducin and PDE from ROS. Unilluminated ROSs were washed twice in isotonic buffer ([R] = 50 μM) to remove the most soluble proteins. PDE and transducin then could be extracted simultaneously by a wash with hypotonic buffer ([R] = 100 μM). When holoPDE was

needed alone, ROSs were first illuminated and then holoPDE was extracted with hypotonic buffer ([holoPDE] = 0.8−1 μM, 80% purity). After a second wash with hypotonic buffer, TαGTPγS was extracted with isotonic buffer supplemented with 200 μM GTPγS ([TαGTPγS] = 2.5−3 μM, >75% purity). Whenever extraction was performed with hypotonic buffer, the ionic strength was subsequently raised to the level of the isotonic measurement buffer.

Partial Reconstitution of ROS Membranes and Phospholipid Vesicles. Two aliquots of unilluminated ROSs were washed in isotonic buffer ([R] = 50 μM) to remove soluble proteins. Pellets 1 and 2 were resuspended ([R] = 25 μM) in isotonic buffer and in isotonic buffer supplemented with 4 M urea, respectively. After a 15-min incubation at room temperature and centrifugation, the ROS membranes were washed in isotonic buffer at a very low membrane concentration ([R] = 1.25 μM) at least twice. This procedure removes about 90% of transducin and PDE (Catty et al., 1992; our unpublished data) from pellet 1 and urea from pellet 2. The final pellets were illuminated and resuspended in a transducin + PDE extract. These suspensions typically contained 25 μM R*, ca. 200 nM PDE, and 1 μM transducin; they were kept in the dark, on ice before use. Large unilamellar vesicles were prepared from azolectin according to Szoka and Papahadjopoulos (1978), as described in Otto-Bruc et al. (1993).

Measurements of cGMP Hydrolysis by Native and Partially Reconstituted ROS Membranes. This was accomplished with the classical pH metric method. The stirred 350-μL sample resided in a thermostated (25 °C) Plexiglas cuvette, which was gently flushed with argon throughout each experiment. The pH electrode, electronics, data acquisition, and processing were identical to those of the stopped-flow/fast filtration setup.

RESULTS

Deactivation of TαGTP-Stimulated holoPDE on Phospholipid Vesicles. In this experiment (Figure 2), we endeavor to recreate the minimal molecular conditions that characterize the *in vivo* interaction between Tα and its effector: (i) binding of Tα to the entire PDE complex, comprising catalytic as well as inhibitory moieties, and (ii) localization of this interaction on the surface of a lipid bilayer. From the stopped-flow/fast filtration apparatus, 700 μL of TαGTP was injected into the pH measurement cuvette, which already contained cGMP and phospholipid vesicles reconstituted with holoPDE, in a volume of 500 μL. The reconstituted holoPDE had a typical basal activity of 2 cGMP/PDE/s. The resulting slight acidification of the preinjection solution between the addition of cGMP and the injection of Tα was neutralized by adding 0.4−0.8 mM KOH to the cuvette. This helped minimize the pH jump at the start of the experiment.

Once the injection was complete, the cuvette contained 50 nM Tα, 200 nM holoPDE, 2 mM cGMP, and 1.5 mg/mL vesicles in a volume of 1.2 mL. These protein and vesicle concentrations were chosen to achieve two crucial molecular interactions. First, the high concentration of vesicles ensured that holoPDE stayed membrane-bound throughout the experiments. Second, the large excess of holoPDE over injected TαGTP ensured that the latter was completely associated with the former. This ought to be the case, given the 1 nM dissociation constant for the TαGTPγS−holoPDE complex (Heck & Hofmann, 1993). Moreover, with this 4:1 holoPDE:Tα stoichiometry, the activated PDE complexes must bear only one TαGTP. For such complexes, Bennett and Clerc (1989) reported a turnover number of 100 cGMP/PDE/s. If the concentration of these active PDE complexes were considered to be equal to that of injected TαGTP, i.e., 50 nM, the expected hydrolysis rate would be 5 μM cGMP/s, which compares well with the maximal rate of 4 μM/s observed in this experiment.

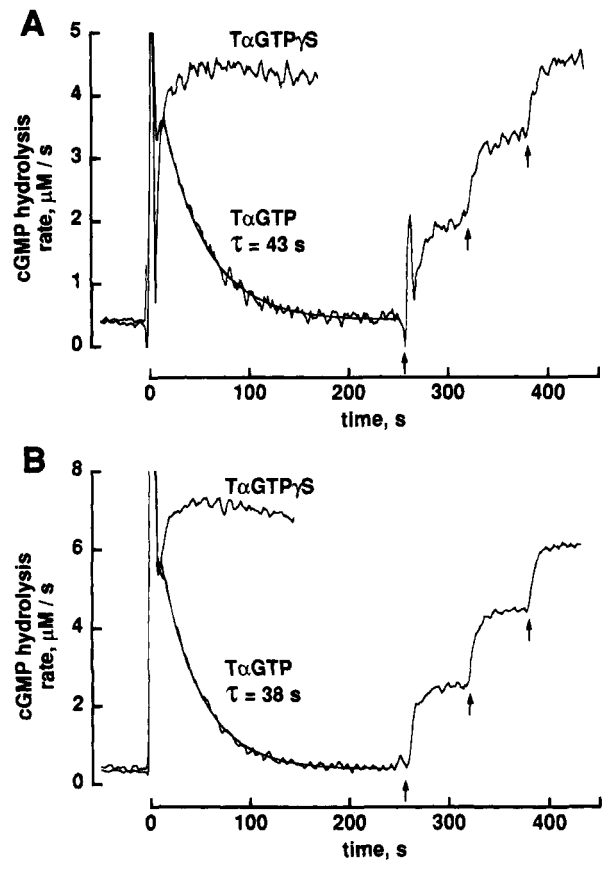

FIGURE 2: (A) Deactivation of Tα-stimulated holoPDE reconstituted on phospholipid vesicles. The cuvette initially contained cGMP and vesicles bearing holoPDE in 500 μL. At zero time, 700 μL containing 85 nM TαGTP was injected into the cuvette from the stopped-flow/fast filtration apparatus. In the final volume of 1.2 mL, there was 1.5 mg/mL vesicles bearing 200 nM holoPDE, 2 mM cGMP, and 50 nM TαGTP. The decay in the rate of cGMP hydrolysis (noisy trace) can be fit with a single-exponential decay (smooth trace) of 43 s. Once this decay was over, three injections of 17 nM TαGTPγS each yielded three equal and stable plateaus of cGMP hydrolysis. When the stream from the stopped-flow/fast filtration apparatus bore TαGTPγS instead of TαGTP, cGMP hydrolysis did not decay but went on as a plateau until the exhaustion of cGMP. (B) Effect of T$\beta\gamma$. When the same experiment as that in part A was repeated with T$\beta\gamma$ (240 nM in the initial solution) added, similar kinetics was observed (decay time of 38 s), but the turnover number of the holoPDE was increased by about 2-fold. These experiments were repeated twice, each using a different batch of vesicles + holoPDE and consisting of duplicate trials. The resulting traces are essentially identical.

The population of active PDE, as measured through the rate of cGMP hydrolysis, decays exponentially with a time constant of ca. 40 s. This deactivation strictly requires GTP hydrolysis, as the control with TαGTPγS showed no decay in this time range. Moreover, it is not due to the exhaustion of cGMP because subsequent injections of TαGTPγS still resulted in cGMP hydrolysis. Once the decay was complete, injection of GTPγS into the cuvette did not cause a

reactivation of the PDE (data not shown). This crucial control shows that the cuvette was not contaminated with minute R*-bearing membrane fragments from the stopped-flow/fast filtration apparatus. Such contamination would have meant that the injected Tα did not turn over just once and that the 40-s decay observed was due to some complex cycling. Clearly, this did not happen: the injected Tα hydrolyzed its bound GTP in one round and could not reload for another as there was no R* in the cuvette.

Kutuzov and Pfister (1994) reported that TαGDP can activate holoPDE, albeit with a very low apparent affinity of 15 μM, and that such activation is abolished in the presence of Tβγ. This implies that once GTP hydrolysis by Tα is complete, Tβγ is needed to recombine with TαGDP and truly terminate all activation of holoPDE. In our experiments, if Tβγ were also present on the vesicle surface, would the deactivation of Tα-stimulated holoPDE be faster? In the experiment of Figure 2B, vesicles were reconstituted with 240 nM Tβγ (i.e., 100 nM after Tα injection) in addition to the usual holoPDE. This presence of Tβγ did not appreciably shorten the deactivation time of holoPDE. On the other hand, it caused an almost 2-fold increase in the rate of cGMP hydrolysis. A molecular explanation for this effect awaits.

GTPase-dependent deactivation of membrane-associated holoPDE therefore occurs in ca. 40 s, a characteristic time that is reminiscent of the 20-s deactivation of soluble, isolated TαGTP (Antonny et al., 1993). In native ROS, the deactivation of membrane-associated PDE also depends on GTP hydrolysis, but it occurs in only 1 s (Vuong & Chabre, 1991). This set of results speaks very clearly against three propositions. (i) Contrary to the interpretation of Arshavsky and Bownds (1992) and Arshavsky et al. (1994), the inhibitory subunit PDEγ is utterly incapable of accelerating the GTPase activity of Tα, even if interaction between the two partners occurs on a membrane surface in the presence of PDEαβ. (ii) Contrary to the assertion of Pagès et al. (1992, 1993), holoPDE and, in particular, its catalytic moieties also are not endowed with such GTPase-activating power. (iii) Contrary to the proposal by Arshavsky et al. (1994), localization per se of the interaction between Tα and holoPDE on a membrane surface has no detectable influence on the GTPase activity of the former.

Deactivation of Tα-Stimulated holoPDE on Native or Partially Reconstituted ROSs. The major difference between the experiments presented so far and those of Vuong and Chabre (1991) resides in the nature of the membrane support: phospholipid vesicles devoid of all proteins except transducin and holoPDE versus native ROS membranes, which besides rhodopsin harbor several other tightly associated species that can only be extracted with urea (A. Otto-Bruc, unpublished data). In the experiment of Figure 3A, we compared the deactivation kinetics of Tα-stimulated holoPDE on native, isotonically stripped, and urea-washed ROS membranes. The rhodopsin concentration was 25 μM, which was needed to force transducin and PDE to stay on the membrane surface. For the reconstituted samples, the concentrations of transducin and PDE were ca. 1 μM and 200 nM, respectively. The reaction was started by injecting 200 nM GTP into the cuvette. Following a quick rise to a peak, the PDE activities from the native, isotonically stripped, and urea-washed ROS samples decayed in 7, 8, and 28 s (1/e time), respectively. Once the decay was over, GTPγS was added stepwise until maximum PDE activation was reached. Typically, this took ca. 600 nM GTPγS, which was substantially more than the 200 nM GTP injected to

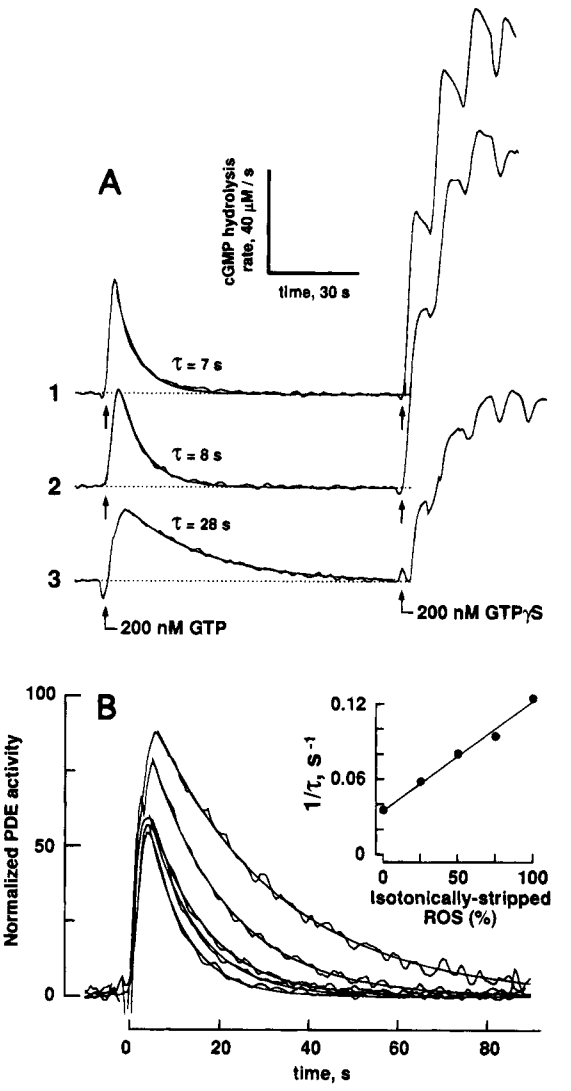

FIGURE 3: (A) Effect of various ROS membrane supports on the deactivation of Tα-stimulated holoPDE. The ROS concentration was 25 μM rhodopsin. The cGMP level was 8 mM. For the reconstituted ROS (traces 2 and 3), the concentrations of reconstituted transducin and holoPDE were 1 μM and 200 nM, respectively. At zero time, 200 nM GTP was injected into the cuvette to start the experiments. At 100 s, when GTP-induced cGMP hydrolysis was complete, three aliquots of 200 nM GTPγS were injected sequentially, which resulted in three comparable plateaus in cGMP hydrolysis. The decay in cGMP hydrolysis was fit with a single exponential (smooth traces). Trace 1: native ROS, deactivation time 7 s. Trace 2: isotonically stripped ROS, deactivation time 8 s. Trace 3: urea-washed ROS, deactivation time 28 s. These experiments were repeated two times and the traces are superimposable. Moreover, experiments using different batches of reconstituted ROS resulted in identical kinetics and comparable amplitudes (±10%). (B) Deactivation of holoPDE from a mixed population of isotonically stripped and urea-washed ROSs. The proportion of isotonically stripped ROS in the five samples was 0, 25, 50, 75, and 100%. The concentrations of rhodopsin, transducin, and holoPDE were kept constant at 25 μM, 1 μM, and 200 nM, respectively. Injections of GTP followed the same protocol as in part A. The data shown were normalized to the cGMP hydrolysis rate induced by 200 nM GTPγS. Fitting the traces yielded deactivation times τ of 28, 17, 12.5, 10.5, and 8 s for the samples containing 0, 25, 50, 75, and 100% isotonically washed ROS, respectively. Inset: the deactivation rate ($1/\tau$) is plotted as a function of the proportion of isotonically stripped ROS.

start the experiment. Hence, one could be pretty well assured that the resulting population of TαGTP became entirely associated with the holoPDE present. The decay observed was thus truly due to just one round of GTP hydrolysis by a population of Tα that was bound to holoPDE.

While with native ROS, this decay took just 7 s, it occurred in 28 s when transducin and holoPDE were reconstituted on ROS membranes that were previously stripped with 4 M urea. Such slowness did not come about because transducin and PDE were reconstituted, because when the procedure was carried out with isotonically stripped ROS, the decay time of 8 s is practically equal to that of native ROS. The culprit is therefore the treatment with urea: some proteinaceous cofactor from the native and isotonically stripped ROSs had been either removed or destroyed *in situ* by 4 M urea. When isotonically stripped ROSs were added to urea-washed ROSs in increasing proportions, while the membrane concentration was kept constant at 25 μM rhodopsin, deactivation of the reconstituted holoPDE decreased from 28 to 8.5 s (Figure 3B). This observation seems to suggest that the GTPase-activating cofactor acts on the Tα-stimulated holoPDE in a catalytic fashion.

Altogether, our results agree with those of Angleson and Wensel (1993), who used the strategy of indirect single turnover to probe the GTPase-dependent inactivation of PDE. At a low ROS concentration (4 μM R*), this rate is low but it can be enhanced by adding hypotonically stripped ROS membranes to the reaction vessel. On the other hand, when the direct single-turnover technique was applied to a dilute suspension of ROSs, the addition of PDEγ did not speed up the GTP hydrolysis of Tα. Further work by these researchers (Angleson & Wensel, 1994) confirms this finding and indicates that PDEγ can enhance the GTPase of Tα if and only if a high concentration of ROS membranes is available. From these studies, they propose that the GAP is a protein in ROS membranes that is distinct from PDEγ or holoPDE and that its target is TαGTP−PDEγ.

We have chosen to strip transducin and holoPDE from ROS membranes by extensive dilution in isotonic buffer, taking advantage of the rather loose membrane association of transducin and holoPDE (Catty et al., 1992; Bruckert et al., 1992). After such a stripping protocol, ROS membranes exhibited a maximal GTPγS-induced cGMP hydrolysis rate that was less than 10% of the rate observed with native ROS (data not shown). The usual method of stripping is to wash ROS membranes in hypotonic buffer, followed by their resuspension in isotonic buffer. We have noticed that such treatment causes a substantial decrease in the activity of the reconstituted holoPDE, compared to untreated ROS. This is probably due to some irreversible disorganization of the membrane structure, which would adversely affect PDE activity as it is rather dependent on the membrane environment (Malinski & Wensel, 1992). Treatment with urea results in a decrease in PDE activity as well (Figure 3A), which might also reflect an irreversible disruption of the membrane structure.

Apparent Interaction between the GTPase-Activating Cofactor and Persistently Activated TαGTPγS−holoPDE. In native or isotonically stripped ROSs, if one created first a population of persistently active TαGTPγS−holoPDE, would it be capable of tying down some of this putative GTPase-activating cofactor, thus delaying the deactivation of a subsequent population of TαGTP−holoPDE? As seen in Figure 3, there was, in our native ROS samples, enough holoPDE to easily accommodate 400 nM activated Tα. Thus, a sequential injection of 100 or 200 nM GTPγS followed by 200 nM GTP would create first some long-lived TαGTPγS−holoPDE and then a second population of TαGTP−holoPDE, whose deactivation should take longer than usual. With isotonically stripped ROSs, the first injection of 200 nM GTPγS gave a plateau with a slight droop. A second injection of 200 nM GTP elicited a pulse of cGMP hydrolysis whose decay took 17 s, which is significantly slower than the 8 s observed when there was no prior injection of GTPγS. When only 400 nM GTP was injected, the deactivation time did not differ much from the usual 8 s (data not shown); this control indicates that the slow down seen with the second GTP injection did not come about simply because a larger than usual amount of Tα had been activated. This lengthening was lessened when only 100 nM GTPγS was used in the first injection. When this double-injection protocol was applied to urea-washed ROSs, there was no lengthening of the GTP-induced cGMP hydrolysis pulse. These results reinforce the conclusion that a protein cofactor exists in native ROSs that accelerates the GTPase activity of Tα in the active transducin−PDE complex. The slight droop seen with GTPγS is probably due to the accumulation of GMP, which inhibits holoPDE (Bennett & Clerc, 1989; our unpublished data) and acidification of the medium. This droop is most pronounced when the PDE activity is highest (Figure 4A) and almost nonexistent at low PDE activity (Figure 2A).

DISCUSSION

Just what exactly does holoPDE do to the GTPase activity of the TαGTP that is bound to it during activation? Providing a clearcut answer to this simple question has been hampered by a technical difficulty: R*, and hence illuminated ROS membranes, is always required to load GTP onto transducin. Having ROS membranes in the reaction vessel means also having all of the tightly associated membrane proteins other than rhodopsin that such membranes inevitably carry. The direct single-turnover technique of Arshavsky et al. (1994), elegant as it is, cannot get around the need for illuminated ROS membranes. The fact that the addition of PDEγ or holoPDE to such membranes causes an increase in the GTPase rate of Tα cannot be given the obvious interpretation that PDEγ by itself or in association with PDEαβ acts as a GTPase-activating protein. Indeed, with just this one observation in hand, one ought not dismiss the alternative interpretation: The increase in the GTPase rate by Tα upon PDEγ addition occurs because these two proteins form a complex that is subject to GTPase activation by a third protein cofactor. Such an increase is only apparent. To effectively choose between these two equally plausible interpretations, one needs to insulate the molecular interaction between TαGTP and holoPDE from the other proteins of ROS membranes. Two requirements are called for: (i) obviously, ROS membranes must not be present in the vessel where GTP hydrolysis by Tα is measured and (ii) a membrane support devoid of all other proteins except holoPDE must substitute for the usual ROS membranes. The former requirement is satisfied with the stopped-flow/fast filtration apparatus, which furnishes a stream of extemporaneously purified TαGTP that is physically separated from the activating ROS membranes. With this device, we

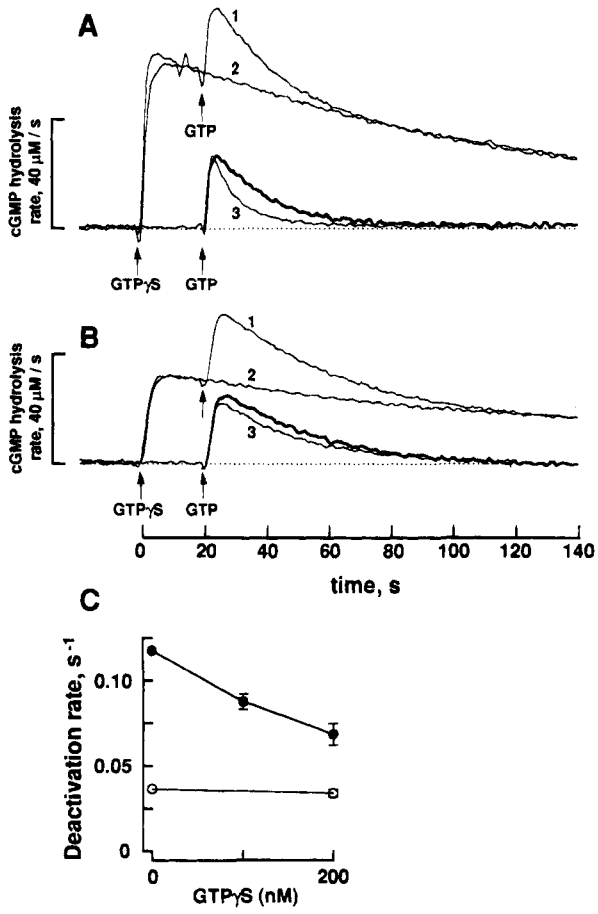

FIGURE 4: Double injections with GTPγS and GTP. The ROS concentration, cGMP level, and concentrations of reconstituted transducin and holoPDE were as for Figure 3. (A) Isotonically stripped ROS. In the first experiment (trace 1), an injection of 200 nM GTPγS at zero time caused a plateau of persistent cGMP hydrolysis. At 20 s, a subsequent injection of 200 nM GTP elicited a cGMP hydrolysis pulse that rides on top of the GTPγS-induced plateau. This plateau is not perfectly stable over the time range of minutes, as can be seen in the second experiment (trace 2) where a sole injection of 200 nM GTPγS was performed at 0 s. This drooping plateau catches up with trace 1 from about 80 s onward. In the third experiment (trace 3), there was just one injection of 200 nM GTP at 20 s. The cGMP hydrolysis pulse shows the usual decay time of 8 s. The kinetics of the cGMP hydrolysis pulse that occurs subsequent to the first injection of GTPγS is obtained by subtracting trace 2 from trace 1. The resulting pulse (thick trace) decays in 17 s, which is significantly slower than the time of 8 s for the control (trace 3). (B) Urea-washed ROS. The same protocol as for part A was carried out. The cGMP hydrolysis pulse that occurred following the GTPγS injection (thick trace) is very similar to the control (trace 3), both decaying in about 30 s. (C) The deactivation rate of the GTP-induced cGMP hydrolysis pulse is plotted as a function of the [GTPγS] used in the first injection. For the isotonically stripped ROS (●), this rate decreases as the GTPγS level grows from 0 to 200 nM, while for the urea-washed ROS (○), it is unchanged. The error bars correspond to extreme values from two aliquots. Two experiments consisting of two aliquots were performed using two different batches of reconstituted ROS. Results from the two experiments are essentially the same.

measured the deactivation of the PDEγ−TαGTP complex in solution and found that association with PDEγ has no effect on the GTPase rate of Tα (Antonny et al., 1993).

These experiments, however, did not truly address the second requirement, in that they were all carried out in solution. In the present work, phospholipid vesicles were used as the membrane support on which holoPDE was reconstituted. Such membrane-supported holoPDE was

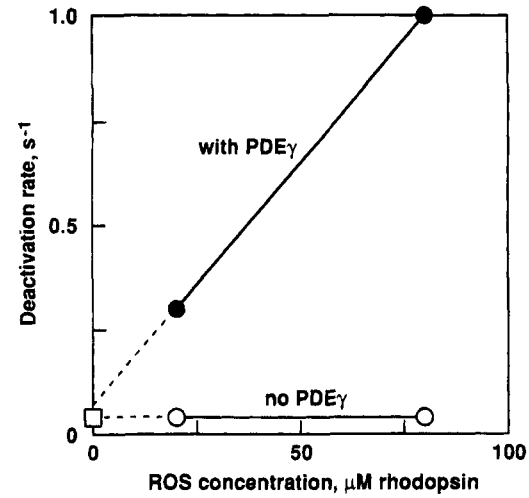

FIGURE 5: Extrapolation to zero [ROS] of the deactivation rate of transducin as measured by the direct single-turnover method. The data reported by Arshavsky et al. (1994) for the deactivation rates of transducin in the presence (●) and absence (○) of PDEγ are shown as a function of ROS concentration (40 and 80 μM). Extrapolation to zero ROS concentration (dotted lines) gives values that are similar to the deactivation rate of soluble TαGTP−PDEγ (□) as measured by Antonny et al. (1993).

efficiently activated by TαGTP supplied by the stopped-flow/fast filtration apparatus, but its GTPase-dependent deactivation took 40 s, a time that resembles the 20-s deactivation kinetics of soluble, unaided TαGTP. Moreover, this slow deactivation could not be speeded up if Tβγ was also present on the phospholipid vesicles. Thus, when one takes the complication caused by the ROS protein complement away from the interaction between TαGTP and holoPDE, but leaves only the strict requirements of a membrane support as well as the availability of Tβγ, holoPDE is utterly incapable of any GTPase-activating influence on the bound Tα. The inability of PDEγ to increase the GTPase activity of Tα can also be discerned in the recent work by Arshavsky et al. (1994), where the effect of increasing the ROS concentration was studied. In Figure 5, we plot the GTPase rate reported by these workers as a function of ROS concentration. When this plot is extrapolated to zero membrane concentration, the GTPase rate obtained is the low value of 0.07 s^{-1}, in spite of the presence of PDEγ in the reaction vessel. This value of 0.07 s^{-1} is very similar to the 0.05 s^{-1} we obtained for soluble Tα, with or without PDEγ (Antonny et al., 1993). In other words, if one could apply the single-turnover technique of Arshavsky et al. (1994) in the limit of no ROS membranes, one would observe a low GTPase rate for Tα whether PDEγ was present or not. From these data, it seems unjustified to bestow the title of GTPase-activating protein on PDEγ.

When holoPDE and transducin (both α- and βγ-subunits) are reconstituted on ROS membranes that have been stripped with 4 M urea, the GTPase-dependent deactivation of holoPDE is drastically slowed compared to a control with native or isotonically stripped ROSs. This is further proof that neither part of holoPDE nor the entire tetramer itself can suffice to enhance the GTPase rate of Tα. In addition, this result shows that Tβγ is also incapable of any GAP action. To some extent, this reconstitution using urea-washed ROS is even more meaningful than the experiments with phospholipid vesicles: the lipid makeup of the membrane is rigorously the same as that in the control, and the main

protein component, namely rhodopsin, is maintained. The only significant difference is the urea treatment, which must destroy membrane proteins other than rhodopsin. Therefore, besides reinforcing the negative finding concerning the role of holoPDE, this reconstitution experiment points to the existence of a protein cofactor that recognizes the stimulated holoPDE and activates the GTPase of the associated Tα. This conclusion is made more credible by the double-injection experiments. A first injection with GTPγS creates a population of persistently active holoPDE, and a second injection with GTP elicits a cGMP hydrolysis pulse that is lengthened compared to the control where no prior activation with GTPγS takes place. Moreover, this lengthening is directly proportional to the [GTPγS] used. We interpret these results as pointing to a significant interaction between the persistently active TαGTP−holoPDE complex and the putative GTPase-activating cofactor. Recently, Angleson and Wensel (1994) reported that the target for this cofactor is TαGTP−PDEγ and not the entire TαGTP−holoPDE. This result does not square with the report of Arshavsky and Bownds (1992), where it can be seen that both species are targets. While our experiments do not explicitly address this issue, our results do suggest that TαGTP−holoPDE is the target of the GTPase-activating cofactor. From the viewpoint of physiology, such a target appears more likely than TαGTP−PDEγ, since *in vivo*, PDE activation does not call for the physical dissociation of TαGTP−PDEγ from the catalytic PDEαβ.

In conclusion, the data presented here, as well as those from Arshavsky et al. (1994) and Angleson and Wensel (1993, 1994), point very strongly to the existence of a GTPase-activating cofactor, which is tightly bound to ROS membranes and is capable of increasing the GTPase rate of Tα in the active holoPDE complex. It would have been very satisfying if holoPDE—or just its inhibitory subunit—had proved sufficient in activating the GTPase activity of the associated Tα (Bourne & Stryer, 1992). Such a hypothesis, so appealing in its elegant simplicity, has not been borne out by subsequent experiments.

ACKNOWLEDGMENT

We are grateful to Nordine Belmokhtar for the design and construction of the stopped-flow/fast filtration apparatus and the pH measurement cuvettes. We thank Marc Chabre for helpful discussions.

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