Enhancement of Rod Outer Segment GTPase Accelerating Protein Activity by the Inhibitory Subunit of cGMP Phosphodiesterase*

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The cGMP phosphodiesterase (PDE) of retinal rod outer segments (ROS) is activated by the GTP-bound form of the G protein, transducin (Gt). This activation can be reversed by the inhibitory subunit of PDE through two distinct mechanisms: acceleration of GTP hydrolysis and direct inactivation independent of GTP hydrolysis. We have found that acceleration of Gt-GTP-PDE activity by PDE does not occur upon formation of a Gt-PDE complex but rather reflects enhanced activity toward this complex of a membrane-bound GTPase accelerating protein. GTPase rate constants for Gt in the presence of 5.3 µM PDE were as high as 0.7 s⁻¹ with hypotonically washed ROS membranes at 40 µM rhodopsin but were more than 10-fold lower when protein-free vesicles containing ROS lipids were substituted for ROS membranes. Acceleration of Gt-GTPase by PDE was also barely detectable at low ROS concentrations (e.g., 4 µM rhodopsin) or if ROS treated with trypsin or urea were used. GTPase-independent inactivation by PDE occurred efficiently at much lower membrane concentrations. Inhibition of Gt-activated PDE was much slower than inactivation of PDE by PDE. Effects of PDE upon successive additions of GTP suggested formation of a complex of PDE and Gt-GDP that is refractory to reactivation.

The G protein transducin (Gt) and its effector, cGMP phosphodiesterase (PDE), play key roles in both the rising and falling phases of the light response of vertebrate photoreceptors (for reviews, see Stryer (1991), Lagrange and Baylor (1992), and Pugh and Lamb (1993)). Electrophysiological recordings of isolated photoreceptors demonstrate that the entire cellular response to light can occur in less than a second (Baylor et al., 1989) suggesting that both activation and inactivation of Gt-PDE occur on this time scale.

Activation of Gt and PDE is initiated by photo-activated rhodopsin (R*) which catalyzes exchange of GTP for GDP on Gt, leading to active GTP-bound Gt-Gt-GTP then activates PDE by relieving the inhibitory constraint of its PDE subunit (Hurley and Stryer, 1982). In vitro studies have demonstrated that Gt activation by GTP uptake and release of Gt from R* occurs in the 100-ms range (Kühn et al., 1981) and that the subsequent interaction between PDE and Gt occurs in less than 5 ms (Heck and Hofmann, 1993). The precise mechanism of Gt-GTP activation of PDE and the molecular composition of the active species are not known with certainty. The results of some studies have been interpreted as indicating that Gt-GTP physically removes PDE (Yamazaki et al., 1983; Wensel and Stryer, 1986; Deterre et al., 1986; Fung and Griswold-Prenner, 1989), but there is mounting evidence that Gt-GTP-PDE, primarily remains in a complex with the catalytic α subunits of PDE during activation (Gray-Keller et al., 1980; Clerc and Bennett, 1992; Catty et al., 1992; Otto-Bruc et al., 1993; Heck and Hofmann 1993). Arshavsky et al. (1992) propose a mechanism in which both forms are important. Efficient activation of PDE by Gt has been found to require the presence of membranes (Fung and Nash, 1983; Tyrinski and O'Brien, 1984; Bennett and Clerc, 1989; Clerc and Bennett, 1992; Malinski and Wensel, 1992), but the membrane enhancement does not require the presence of other membrane-associated proteins (Malinski and Wensel, 1992).

Electrophysiological recordings of the light response in photoreceptors have indicated that PDE inactivation occurs rapidly (<2 s) in intact rod cells (Hodgkin and Nunn, 1988). Biochemical measurements of PDE inactivation (Vuong and Chabre, 1991) and Gt GTPase (Arshavsky et al., 1989; Dratz et al., 1987) in concentrated suspensions of ROS also demonstrated relatively fast rates of inactivation. However, Gt GTP hydrolysis and PDE inactivation are much slower (tens of seconds) in dilute ROS (Angleslon and Wensel 1993) and references therein). Several recent studies indicate that this discrepancy may be accounted for by acceleration of Gt-GTPase and/or PDE inactivation by various components of ROS. Gt GTPase has been found to be accelerated by PDE (Arshavsky and Bownds, 1992; Pages et al., 1992; Angleslon and Wensel, 1993) as well as by a membrane-associated protein distinct from PDE (Angleslon and Wensel, 1993). Studies addressing regulation of Gt-GTPase by PDE, have been less consistent. PDE, has been reported to accelerate Gt-GTPase (Arshavsky and Bownds, 1992), to have no significant effect by itself on Gt-GTPase (Pages et al., 1992; Antony et al., 1993; Angleslon and Wensel, 1993), and to inhibit Gt-GTPase (Yamazaki et al., 1993; Pages et al., 1993). PDE, has also been found to accelerate inactivation of PDE independently of GTP hydrolysis by Gt-GTPase (Erickson et al., 1992; Angleslon and Wensel, 1993).

The experiments described here address the interactions between PDE and Gt involved in regulating both Gt-GTP hydrolysis and GTPase-independent PDE inactivation and also provide information concerning the nature of the active form of PDE.

MATERIALS AND METHODS

Preparation of Rod Outer Segments and Proteins—ROS were isolated under dim red light from frozen dark-dissected bovine retinas (J. A.
PDE was purified from salt extracts of bleached ROS on a DE-52 column (Whatman) as described (Baeher et al., 1979). PDE, was reconstituted into phospholipid vesicles by reverse-phase partition chromatography as described (Wensel and Stryer, 1990) or expressed in Escherichia coli as a recombinant PDE, fusion protein (Brown and Stryer, 1989; Brown 1992). Recombinant PDE, was used for experiments with concentrated ROS membranes. Both forms of PDE, were used in experiments with dilute ROS, and the two forms gave similar results for both GTPase activation and PDE inhibition in those experiments.

Preparation of Washed or Proteolyzed ROS Membranes—Hypotonically washed ROS membranes were prepared by homogenization of bleached ROS in 5 mM Tris (pH 7.4), 2 mM MgCl₂, 50 mM dithiothreitol, followed by centrifugation to pellet the membranes. The washing procedure was repeated at least 3 times. GTP-S-washed ROS membranes were prepared by including an additional washing step, which contained GTP-S at a concentration greater than GTP or GTPyS followed by a series of washes to remove free GTP-S. ROS membranes stripped with 4 M urea were prepared under dim red light as described (Yamamoto et al., 1985).

Trypsinized membranes were prepared by incubating hypotonically washed ROS membranes at a concentration of 5 μM for 90 min at room temperature with either trypsin (130 μg/ml) or premixed trypsin and soybean trypsin inhibitor (130 and 900 μg/ml, respectively). At the end of the reaction, soybean trypsin inhibitor was added to the reaction mixture, and both samples were put on ice and centrifuged to concentrate the membranes. Traces of residual PDE activity in the membranes were typically less than or equal to 1 μM PDE/40 μM GTP. The activity was not increased by stimulation with GTPγS. To ensure that the trypsin in each sample was completely inhibited, an aliquot of each sample was added to PDE, which was then assayed for PDE activity. The aliquots were unable to activate PDE detectably (data not shown), confirming that the trypsin was fully inhibited.

Preparation of Phospholipid Vesicles—Total ROS phospholipids were extracted from ROS membranes in 2:1 (v/v) chloroform:methanol. The ROS phospholipids were dissolved at a 1:1 ratio with phosphatidylcholine (Avanti) to promote bilayer phase vesicles instead of hexagonal phase aggregates that have been reported to be formed by protein-free ROS phospholipids (De Grip et al., 1979). The lipids were then dried under a gentle stream of N₂ and residual organic solvent was removed under reduced pressure overnight. The dried phospholipid film was resuspended in 10 mM Tris, pH 8.0, 0.2 mM MgCl₂, 50 mM NaCl, 0.05% NaN₃ (w/v). The suspensions of phospholipid vesicles were then sonicated 6 times for 30 s on ice using a Branson 185 Sonifier with a micro tip at a setting of 7. The phospholipid concentration was determined by assay of phosphate (Chen et al., 1956) as described (Malinski and Wensel, 1992). Concentrations of the various nucleotide stocks, which were stored at −80 °C, were determined spectrophotometrically.

Data Analysis—The change in pH due to cGMP hydrolysis by PDE (Lieberman and Evaszenak, 1982) was used to monitor PDE activity as described previously (Angleton and Wensel, 1993). PDE activity is reported or plotted as the derivative of cGMP hydrolysis with respect to time derived from a continuous record of pH. The assays were adjusted to 10 or 25 mM Tris, pH 8.1, 2 mM MgCl₂, 100 mM NaCl, and 50-100 μM adenylyl-imidodiphosphate (AMP-pNP). GTP or GTPyS and cGMP were present at the indicated concentrations. Nonlinearity of the assays due to GTP depletion, product inhibition, and change in pH was negligible as determined by methods described previously (Angleton and Wensel, 1993). Stock solutions of nucleotides or proteins added to PDE assays were carefully adjusted to the pH of the assay buffer in order to minimize pH changes not caused by GTP hydrolysis, and cGMP was taken to avoid disturbing the electrode during additions. All assays were performed at 23 °C with fully bleached rhodopsin.

Single Turnover GTPase Assays—GTPase assays were performed under the same conditions as PDE assays, except for dark experiments, described below. GTP hydrolysis was monitored using an acid quench protocol and was converted to [γ-32P]GTP to [α-32P]GDP under single turnover conditions (total GTP < total GTP, as described previously (Angleton and Wensel, 1995).

Verification of Single Turnover Conditions—Control experiments and analysis demonstrating that values for the GTP hydrolysis rate constant, kcat, are independent of the type of GTP used (from GTPγS) and that GTPγS and GTP bind GTP with high affinity (K₅0), although the concentration of the latter clearly affects uptake kinetics. Our analysis of single turnover GTP hydrolysis is also supported by previous reports that the GTP, activation-inactivation cycle is not limited by the activation phase when measured with [GTP] above 10 μM (Guy et al., 1995).

To test for GTPase activity not due to GTPγS, experiments were conducted in the dark with unbrightened ROS and 30 mM hydroxylamine. Independent of the ROS concentration and the presence or absence of PDE, the GTPase activity under dark conditions was approximately ten-times that observed in the same samples after illumination, and PDE, did not enhance the dark GTPase activity (data not shown). All reported experiments were conducted by filtering experiments conducted under similar conditions to those of our GTPase measurements confirmed that within the time resolution of such experiments (~10 s under our conditions), the levels of free GTP remaining during the hydrolysis time course were insignificantly low (data not shown).

GTP hydrolysis by GTPγS and inactivation of PDE occurred with similar kinetics when assayed in the absence of added PDE, at any concentration of ROS membranes in those experiments (from 25 to 250 μM PDE).

Membrane Requirement for Acceleration of G, GTPase Activity—GTPase activity was re-
brane concentrations (40 PM R) still supported potent GTPase acceleration in the presence of dilute ROS (6 pM R) and 1 pM PDE, (Fig. 2D).

Additional experiments using membranes washed with GTPγS to deplete G, showed that soluble G, subunits are also not responsible for the GAP activity. The G, depleited membranes (40 μM R) still supported potent GTPase acceleration when mixed with dilute ROS (6 μM R) and 1 μM PDE, (Fig. 2D).

This result also demonstrates the ability of added membranes to affect GTPase of G, originating on distinct membranes, because even traces of membrane-bound G, remaining in the added GTPγS-stripped membranes were in the G,,-GTPγS form. This ability of added membranes to affect GTPase of G, originating on the dilute ROS may be due to the solubilization...
Membrane Requirement for Acceleration of $G_{\alpha}$ GTPase

Addition of PDE$_\alpha$ as part of holo-PDE (PDE$_{h\alpha}$) gave only ~20% of the acceleration observed with equimolar PDE, in the free form (Fig. 3). The holo-PDE was able to interact functionally with G$_\alpha$ on the membrane surface as demonstrated by its ability to be activated by G$_\alpha$-GTP$_\gamma$S in a membrane-dependent manner (data not shown). We have previously reported (Angleson and Wensel, 1993) that addition of micromolar amounts of PDE to even higher concentrations of ROS membranes (up to 137 $\mu$M) still does not result in inactivation rates comparable with those induced by adding free PDE (Fig. 2). Thus, efficient GAP enhancement by PDE, likely requires its dissociation from PDE.

**GTPase-independent Effects of PDE, on Inactivation of G$_\alpha$ and PDE—**Although high membrane concentrations were required for significant effects of PDE, on GTPase, they were not needed for a GTPase-independent process of inactivation induced by PDE., When PDE activity and G$_\alpha$ GTPase were monitored in parallel experiments in dilute ROS (4 $\mu$M) with exogenous PDE, the PDE inactivation rate was significantly faster than the G$_\alpha$ GTP hydrolysis rate (Fig. 1), demonstrating GTPase-dependent PDE inactivation as previously reported (Angleson and Wensel, 1993; Erickson et al., 1992). Under these conditions, G$_\alpha$ GTPase was only slightly affected (26% increase) by the addition of 500 nM PDE, (Fig. 1). The GTPase-independent effects of PDE, on inactivation are easily distinguished from the GTPase-dependent effects by the use of the hydrolysis-resistant analogue GTP$_\gamma$S (Fig. 4). Assays conducted at an ROS concentration of $10 \mu$M R showed essentially no inactivation of G$_\alpha$-GTP$_\gamma$S-activated PDE in the absence of exogenous PDE, (Fig. 4A). However, when 235 nM PDE, was added to the ROS prior to activation by addition of 150 nM GTP$_\gamma$S, the peak level of PDE activity was greatly reduced, and after reaching a peak, the activity declined to the basal level with a rate constant of 0.10 s$^{-1}$ (Fig. 4A). When successive additions of GTP$_\gamma$S were made in the presence of excess PDE, different results were obtained upon each addition (Fig. 4B). The second GTP$_\gamma$S addition elicited a peak level of PDE activity essentially identical to the first addition, but the subsequent inactivation occurred almost 5-fold more slowly. A third addition of 150 nM GTP$_\gamma$S did not measurably activate the PDE, although the PDE displayed full catalytic activity when treated with trypsin (Fig. 4B). Thus, in these experiments, as in many other similar ones, we observed a persistent inactivation of G$_\alpha$.

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**Fig. 3. Comparison of GAP stimulation by PDE, in the presence and absence of catalytic subunits.** Time course of GTP hydrolysis in hypotonically washed ROS membranes (40 $\mu$M R, 2.1 $\mu$M G$_\alpha$, 0.03 $\mu$M PDE) with no added proteins (□) or 500 nM PDE, added either bound to PDE (■) (250 nM PDE$_{h\alpha}$) or without catalytic subunits (△). Rate constants for single exponential curves drawn are as follows: 0.056 s$^{-1}$ (no added proteins); 0.063 s$^{-1}$ (250 nM added PDE$_{h\alpha}$); 0.38 s$^{-1}$ (500 nM added PDE). This experiment is representative of three separate similar experiments that directly compared the potency of PDE, and PDE$_{h\alpha}$, in GTPase acceleration.

**GAP enhancement by PDE, likely requires its dissociation from PDE.**

**Fig. 4. PDE, inactivation of G$_\alpha$-GTP$_\gamma$S-activated PDE.** A, time course of PDE activity elicited by 150 nM GTP$_\gamma$S in ROS (10 $\mu$M R, 300 nM G$_\alpha$, 50 nM PDE) with no added PDE, (upper trace). Values of peak activities are 74.0 $\mu$mol cGMP s$^{-1}$ for no added PDE, sample and 10.8 $\mu$mol cGMP s$^{-1}$ for the sample with added PDE. Initial cGMP concentrations were 4 nM for the sample with added PDE, and 3 nM for the sample with no added PDE, B, PDE activity in the sample of ROS with 235 nM added PDE, shown in A, on a longer time scale and plotted as a percent of the activity in the sample with no added PDE, First three arrows indicate times of addition of 150 nM GTP$_\gamma$S, and last arrow indicates addition of trypsin (0.17 mg/ml), which elicited full PDE activity. The traces shown are representative of at least six separate assays that all demonstrated similar effects of PDE, on GTP$_\gamma$S-G$_\alpha$-activated PDE.

**The slower inhibition upon the second addition of GTP$_\gamma$S may be explained partly by depletion of the initial PDE pool, but it is not clear whether the entire time course can be readily explained by a single second order inhibition reaction. To observe the inhibition kinetics without interference from activation kinetics, we preincubated GTP$_\gamma$S and ROS, and, after an equilibrium level of PDE activity was attained, we added PDE, (Fig. 5). The time course of inactivation was fit as a second order process with a rate constant of $2 \times 10^4$ M$^{-1}$ s$^{-1}$ (see "Data Analysis" under "Materials and Methods"). At least part of the effect of PDE, is presumably due to formation of a complex with G$_\alpha$-GTP$_\gamma$S, given the sub-nanomolar $K_d$ reported for formation of this complex (Otto-Bruc et al., 1993), but it is not clear whether the inhibition observed is due to dissociation of G$_\alpha$-GTP$_\gamma$S from PDE, with subsequent sequestration by PDE, or due to direct action on the G$_\alpha$-PDE complex. That one or both of these processes is likely to be involved can be concluded from the observation that the rate constant for inhibition observed here ($<10^3$ s$^{-1}$) is approximately twice orders of magnitude slower than the near-diffusion-limited rate constants (on the order of $10^5$ M$^{-1}$ s$^{-1}$) for binding of PDE, to either PDE$_{h\alpha}$ or to G$_\alpha$-GTP$_\gamma$S (Wensel and Stryer, 1986, 1990; Brown, 1992; Otto-Bruc et al., 1993).

The results obtained when added PDE, was present during activation by GTP are consistent with those obtained with...
membrane requirement for acceleration of G \textsubscript{ta} GTPase

**FIG. 5. Kinetics of inhibition of G \textsubscript{ta}-GTP\textsubscript{y}S-activated PDE by PDE\textsubscript{y}.** PDE\textsubscript{y} PDE in ROS (10 \mu m R, 300 \text{ nM} G\textsubscript{a}, 50 \text{ nM} PDE) was activated by 150 \text{ nM} GTP\textsubscript{y}S to a PDE activity of 71.2 \mu M cGMP s\textsuperscript{-1} (100%). PDE\textsubscript{y}(235 \text{ nM}) was then added at the zero time point. The curve is a fit to the data using the second order reaction scheme described under "Materials and Methods" and a value of \( k_2 = 2.4 \times 10^8 \text{ M}^{-1} \text{s}^{-1} \). This trace is representative of several similar assays for which \( k_2 = 2.3 \pm 0.4 \times 10^8 \text{ M}^{-1} \text{s}^{-1} \) (n = 4).

GTP\textsubscript{y}S; lower peak PDE activities and accelerated inactivation kinetics (i.e., faster than GTP hydrolysis) were consistently observed (Fig. 6). At low membrane concentrations (Fig. 6A), the first GTP addition gave rise to a robust, but diminished, activation of PDE, with rapid inactivation. Successive GTP additions gave rise to progressively lower levels of peak activity and slower inhibition, until even 15 \text{ nM} GTP\textsubscript{y}S elicited a barely detectable response. This cumulative lowering of peak activity and slowing of inactivation is consistent with depletion of both the PDE\textsubscript{y} and G\textsubscript{a} pools in an activation-dependent manner as more and more GTP is added. The persistent nature of the inactivation suggests that even after it returns to the GDP form, G\textsubscript{a} remains bound to PDE, and is thus refractory to subsequent rounds of activation. At higher membrane concentrations (137 \mu m R, Fig. 6B), such a refractory PDE\textsubscript{y}-G\textsubscript{a}-GTPyS complex appeared to exist only transiently. Multiple additions of 100 or 200 \text{ nM} GTP showed no signs of depleting PDE, initially present at only 50 \text{ nM}. PDE was consistently activated by GTP to a level that was reduced from that observed without PDE\textsubscript{y}, and the inactivation rate was reproducibly increased by 64% relative to the rate with added PDE\textsubscript{y}. The observation that G\textsubscript{a} and PDE can compete for binding to G\textsubscript{a} (Yamazaki et al., 1990; Otto-Bruc et al., 1993) suggests that higher concentrations of G\textsubscript{a} and of R* may allow formation of the R*-G\textsubscript{a}PDP\textsubscript{y} complex to override formation of PDE\textsubscript{y}-G\textsubscript{a}-GDP.

**FIG. 6. Effects of PDE\textsubscript{y} on activation by GTP in dilute and concentrated ROS.** A, PDE activity in dilute ROS (4 \mu m R, same sample as Fig. 1) with 160 \text{ nM} added PDE\textsubscript{y}. First four solid arrows indicate times of addition of 100 \text{ nM} GTP, the next two dashed arrows indicate additions of 15 \text{ nM} GTP\textsubscript{y}S, and the last arrow indicates addition of trypsin (0.17 mg/ml). PDE activity is plotted as a percent of PDE activity elicited by 100 \text{ nM} GTP in a sample with no added PDE\textsubscript{y} (12.4 \mu M cGMP s\textsuperscript{-1}). 2 mM cGMP was present initially. B, time course of PDE activity in concentrated ROS (137 \mu m R, 5.1 \mu M G\textsubscript{a}, 0.75 \text{ nM} PDE) with 50 \text{ nM} added PDE\textsubscript{y}. The first three arrows show time of addition of 100 \text{nM} GTP and the last two arrows show time of addition of 200 \text{nM} GTP. The PDE activity is expressed as a percent of PDE activity elicited by 200 \text{nM} GTP in a sample with no added PDE\textsubscript{y} (160 \mu M cGMP s\textsuperscript{-1}). 25 mM cGMP was present initially. The inset shows the value of \( k_{\text{act}} \) from assays with no added PDE\textsubscript{y}, (\( k_{\text{act}} = 0.140 \pm 0.006 \text{ s}^{-1} \)), and with 50 \text{ nM} added PDE\textsubscript{y}, (\( k_{\text{act}} = 0.230 \pm 0.018 \text{ s}^{-1} \)).

**DISCUSSION**

**Acceleration of the Single Turnover GTPase Activity of G\textsubscript{a} by PDE\textsubscript{y} is Dependent upon Another Protein Component of ROS—** The results reported here confirm previous reports that free PDE\textsubscript{y} can have dramatic effects, in vitro, on the inactivation of G\textsubscript{a} and PDE by both GTPase-independent mechanisms (Erickson et al., 1992; Angleson and Wensel, 1993) and by accelerating the GTPase activity of G\textsubscript{a} (Arshavsky and Bownds, 1992). GTPase acceleration is not, however, the consequence of simply forming a PDE\textsubscript{y}-G\textsubscript{a}-GTP complex (Antony et al., 1993; Angleon and Wensel, 1990) even in the presence of a high concentration of phospholipid membranes (Fig. 2). Instead, the results reported here demonstrate that an additional membrane protein is required for PDE\textsubscript{y} GTPase accelerating activity. ROS membrane enhancement of PDE\textsubscript{y}-induced GTPase acceleration has also been observed by others in bovine ROS.\footnote{V. Yu. Arshavsky, personal communication.} This effect may be dependent on the membrane-bound protein that has its own GTPase accelerating activity (Angleson and Wensel, 1993) or may depend on yet another factor. The results reported here do indicate that R* is not sufficient for GTPase acceleration and that membranes depleted of G\textsubscript{a} subunits show strong GTPase acceleration (Fig. 2D). These conclusions hold regardless of the presence or absence of PDE\textsubscript{y}.

There are a number of features of the activity of PDE\textsubscript{y} in enhancing GTPase acceleration of G\textsubscript{a} that distinguish the interactions involved from those responsible for activation of PDE by G\textsubscript{a}, or for formation of the G\textsubscript{a}-PDE complex in solution. First, although ROS membranes enhance PDE activation as well as GTPase acceleration, much lower concentrations of ROS membranes are required for maximal stimulation of PDE activation (half-maximal effect at 0.4 \mu m R), and protein-free lipid vesicles are actually somewhat better at stimulating PDE activation than are protein-containing ROS membranes (Malinski and Wensel, 1992). Although G\textsubscript{a}-GTP\textsubscript{y}S-PDE has fairly high affinity for ROS membranes (Malinski and Wensel, 1992; Heck and Hofmann, 1993), the G\textsubscript{a}-GTP\textsubscript{y}S-PDE complex has been shown to have low affinity for ROS membranes (Yamazaki et al., 1990; Catty et al., 1992; Arshavsky et al., 1992). This suggests that the PDE, interaction with protein-containing ROS membranes (40 \mu m R) (Figs. 2 and 3) which supports GTPase acceleration, is a transient and relatively weak interaction. This idea is supported by the observation that only partial GTPase acceleration is observed at an estimated free PDE\textsubscript{y} concentration of 50 nm (Fig. 24), a value 500 times the \( K_{\text{y}} \) value for the PDE\textsubscript{y}-G\textsubscript{a}-GTP\textsubscript{y}S complex in solution (Otto-Bruc et al., 1993).

Although PDE\textsubscript{y} was reported to accelerate G\textsubscript{a} GTPase in amphibian and bovine ROS (Arshavsky and Bownds, 1992;
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Arshavsky et al., (1993), several other studies have indicated that PDE, does not appreciably increase the GTPase activity of $G_{o}$ (Pages et al., 1992; Antony et al., 1993; Angleson and Wensel, 1993), and still other studies have reported that PDE, inhibits $G_{o}$ GTPase (Yamazaki et al., 1993; Pages et al., 1993). The results reported here suggest that these discrepancies can be accounted for by use of different concentrations of membranes containing the GTPase-accelerating factor as well as different methods of GTPase measurement (i.e. steady-state versus single turnover conditions).

Yamazaki et al. (1993) reported that PDE, potently inhibited steady-state GTPase in dilute amphibian ROS membranes (4 μM R) reconstituted with $G_{o}$ and $G_{o}$, and others have reported that PDE, weakly inhibited steady-state GTPase in concentrated suspensions of bovine ROS (Pages et al., 1993). PDE, has been found to bind tightly to $G_{o}$-GDP and can actually remove $G_{o}$-GDP from dark ROS membranes (Yamazaki et al., 1990; Otto-Bruc et al., 1995). It is likely that under steady-state conditions, PDE, remains bound to $G_{o}$-GDP and inhibits its reassociation with $G_{o}$, and R*. The resulting PDE,--$G_{o}$ complex would be inhibited from further participation in multiple rounds of GTP uptake and hydrolysis, thus giving rise to a net inhibition of steady-state GTP hydrolysis. Our results (Figs. 1B and 6A) are consistent with the association-dependent formation of an inhibited PDE,--$G_{o}$-GDP complex, and it has been reported (Arshavsky et al., 1993) that PDE, accelerates $G_{o}$ GTPase under single turnover conditions only and does not accelerate GTPase measured under steady-state conditions.

The possibility of added factors affecting the kinetics of GTP uptake underscores the value of single turnover measurements for monitoring GAP activity and, in general, for determining the kinetics of hydrolysis of GTP already bound to $G_{o}$ (e.g. Arshavsky and Bownds, 1992; Angleson and Wensel, 1993; Antony et al., 1993).

GTPase-independent Effects of PDE,—Drastic effects of PDE, on PDE inactivation can be observed at ROS concentrations at which PDE, does not significantly influence $G_{o}$ GTPase. The fact that PDE, can cause GTPase-independent inactivation can be demonstrated either by directly comparing GTPase rates with PDE inactivating interactions (Fig. 1) (Angleson and Wensel, 1993) or by activating $G_{o}$ with GTP-$\gamma$S (Fig. 4) (Erickson et al., 1992). The mechanism of GTPase-independent inactivation remains to be determined and may involve multiple steps.

Physiological Role of Free PDE,—PDE (the holoenzyme PDE$_{apo}$) does not mimic the action of PDE, on GTPase-independent inactivation (Angleson and Wensel, 1993) or the extent of GTPase acceleration of $G_{o}$ (Fig. 1) (Arshavsky and Bownds, 1992; Angleson and Wensel, 1993). The modest enhancement of $G_{o}$ GTPase by holo-PDE has recently been attributed to the catalytic PDE$_{apo}$ subunits rather than to the PDE, subunits (Pages et al., 1993).

PDE, has high affinity for PDE$_{apo}$ ($K_{d}$, $\sim$10$^{-11}$ M) (Wensel and Stryer, 1986, 1990) as well as for $G_{o}$-GDP ($K_{d}$, 3.0 nM) and can remove $G_{o}$-GDP from dark bovine membranes (Otto-Bruc et al., 1993). These affinities suggest that any PDE, in a rod cell not bound to PDE$_{apo}$ may be complexed with $G_{o}$ (present at $\sim$300 μM) Thus, the physiological relevance of effects exerted by free PDE, in bovine ROS remains uncertain.

The finding that cGMP concentration can regulate the ability of amphibian PDE to accelerate $G_{bo}$ GTPase in amphibian ROS (Arshavsky et al., 1991; Arshavsky and Bownds, 1992) led to the proposal of a mechanism (Arshavsky et al., 1992) for dissociation of a $G_{o}$-PDE complex from holo-PDE during adaptation to background light in amphibian ROS; decreases in cGMP could trigger dissociation of cGMP from the noncatalytic cGMP binding sites on PDE (Yamazaki et al., 1980; Gillespie and Beavo, 1989) causing release from PDE of the $G_{o}$-PDE complex, which would display rapid GTP hydrolysis. Cote and Brunnock (1993) have reported that cGMP dissociates from high affinity sites on frog PDE in times on the order of a few minutes, which is too slow to allow for involvement of these sites in visual excitation and recovery. However, the same report indicates that cGMP dissociates from unidentified sites at sufficient rates to have a possible role in phototransduction. The proposed mechanism for dissociation of $G_{o}$-PDE, from PDE by lowering [cGMP] appears unlikely to apply to bovine ROS, because cGMP dissociation from the noncatalytic sites on bovine PDE has a $t_{1/2}$ of $\sim$4 h at 37 °C (Gillespie and Beavo, 1989). Furthermore, free [cGMP] does not affect the ability of bovine PDE to accelerate GTPase (Angleon and Wensel, 1993). The fact that bovine PDE activated by $G_{o}$, in the absence of cGMP does not behave like PDE, in terms of either GTPase-independent inactivation or GTPase acceleration (Angleson and Wensel, 1993) also suggests that free PDE, is not generated. Thus, for PDE, to affect either GTPase-independent inactivation or $G_{o}$ GTPase, in bovine photoreceptors in vitro, a TGPPase-like molecule or an alternative mechanism for release of PDE, from PDE would have to exist in bovine ROS.

An estimate of the pool size of the membrane-bound GAP can be made from the observation that the effect of bleached membranes on $G_{o}$ GTPase in the absence of PDE, is half-saturated at a membrane concentration of approximately 20 μM R with 100 μM activated $G_{o}$ (Angleson and Wensel, 1993). If $G_{o}$-GTP forms a stoichiometric complex with the GAP, a lower limit for the ratio of rhodopsin to GAP in ROS would be 200:1, suggesting an in vivo concentration of GAP on the order of tens of micromolars. An intriguing question concerns the nature of the PDE, interaction that allows efficient GTPase acceleration by the membrane-bound GAP. Although the most economical explanation of the dual requirement for free PDE, and the GAP is that PDE, induces a conformation of $G_{o}$-apo that interacts more strongly with the GAP, an alternative mechanism could involve the critical interactions between PDE, and the GAP rather than between PDE, and $G_{o}$. Although PDE, forms high affinity specific complexes with $G_{o}$ and PDE$_{apo}$, PDE,apos GAP-enhancing interactions are much weaker. They may reflect some less specific effects of PDE,apos unusual amino acid sequence, which includes distinct stretches of basic and acidic residues. Further characterization of the GTPase accelerating protein will probably be necessary before such questions can be answered.

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REFERENCES
Membrane Requirement for Acceleration of GTPase


