Membrane Stimulation of cGMP Phosphodiesterase Activation by Transducin: Comparison of Phospholipid Bilayers to Rod Outer Segment Membranes†

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ABSTRACT: To clarify the role of phospholipids in G protein–effector interactions of vertebrate phototransduction, transducin activation of cGMP phosphodiesterase (PDE) has been reconstituted on the surface of well-defined phosphatidylcholine (PC) vesicles, using purified proteins from bovine rod outer segments (ROS). PC vesicles enhanced PDE stimulation by the GTP-γS-bound transducin α subunit (T_α-GTPγS) as much as 17-fold over activation in the absence of membranes. In the presence of 3.5 μM accessible PC in the form of large (100 nm) unilamellar vesicles, 500 nM T_α-GTPγS stimulated PDE activity to more than 70% of the maximum activity induced by trypsin. Activation required PC, PDE, and T_α-GTPγS, but did not require prior incubation of any of the components, and occurred within 4 s of mixing. The PC vesicles were somewhat more efficient than urea-washed ROS membranes in enhancing PDE activation. Half-maximal activation occurred at accessible phospholipid concentrations of 3.8 μM for PC vesicles, and 13 μM for ROS membranes. Titrations of PDE with T_α-GTPγS in the presence of membranes indicated a high-affinity (K_{act} < 250 pM) activation of PDE by a small fraction (0.5–5%) of active T_α-GTPγS, as did titrations of ROS with GTPγS. When activation by PC vesicles was compared to PDE binding to membranes, the results were consistent with activation enhancement resulting from formation of a T_α-GTPγS-dependent PDE–membrane complex with half-maximal binding at phospholipid concentrations in the micromolar range. The value of the apparent dissociation constant, K_{PL}, associated with the activation enhancement was estimated to be in the range of 2.5 nM (assuming an upper limit value of 1600 phospholipids/site) to 80 nM (for a lower limit value of 50 phospholipids/site). Another component of membrane binding was more than 100-fold weaker and was not correlated with activation by T_α-GTPγS. Low ionic strength disrupted the ability of ROS membranes, but not PC vesicles, to bind and activate PDE. Removal of PDE's membrane-binding domain by limited trypsin digestion eliminated both the binding of PDE to vesicles and the ability of PDE to be activated by T_α-GTPγS and membranes. These results suggest that ROS membrane stimulation of PDE activation by T_α-GTPγS is due almost exclusively to the phospholipids in the disk membrane.

Transducin and cGMP phosphodiesterase (PDE[1]) are peripheral membrane proteins found on the disk membranes of vertebrate rod cells [for reviews, see Stryer (1986, 1991), Liebman et al. (1987), and Hurley (1987)]. In its active, GTP-bound form, the α subunit of transducin (T_α-GTP) activates PDE by reversing the effect of the PDE inhibitory subunit, PDE_γ (Hurley & Stryer, 1982; Wensel & Stryer, 1986). While T_α-GTP and PDE_γ are known to act in reciprocal fashion to regulate the catalytic subunits ($PDE_{\alpha B}$), the composition and stoichiometry of the complex(es) formed by T_α-GTP and PDE subunits and the relationship of the proteins to the membrane when PDE is activated have yet to be determined with certainty.

The affinity of T_α for membranes is greatly reduced in the GTP form, and light scattering signals from rod outer segments have been interpreted as indicating the rapid release of T_α-GTP from membranes triggered by light activation of rhodopsin (Kühn et al., 1981; Lewis et al., 1984; Vuong et al., 1984). Despite the increased solubility of T_α in the activated form, it only activates PDE efficiently in the presence of membranes (Fung & Nash, 1983; Ho & Fung, 1984; Tyminski & O'Brien, 1984; Wensel & Stryer, 1986; Miller et al., 1987; Bennett & Clerc, 1989; Phillips et al., 1989), implying a requirement for a membrane-bound complex to form activated PDE. While the solubilization of T_α upon binding GTP is unique among characterized G proteins, the localization of G protein–effector interactions at the cytoplasmic surface of membranes is a common characteristic of all known G protein–effector interactions.

In order to determine the molecular mechanism for this membrane-mediated activation, it is essential to be able to reconstitute the activation process from well-defined purified components whose concentrations can be controlled and whose participation in complex formation can be accurately determined. Activation of purified PDE by purified transducin has been reconstituted on membranes, but previous studies have used membranes containing significant amounts of other proteins [primarily rhodopsin; e.g., Fung and Nash (1983), Tyminski and O'Brien (1984), Wensel and Stryer (1986),

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[1] Abbreviations: PDE, photoreceptor cGMP phosphodiesterase, $PDE_{\alpha\beta\gamma\gamma}$; T, transducin, the photoreceptor G protein; T_α and $T_{\beta\gamma}$, α and βγ subunits of transducin; T_α-GTPγS, activated transducin, GTPγS-bound α subunit; PC, phosphatidylcholine; LUV, large unilamellar vesicles, approximately 100 nm in diameter; MLV, multilamellar vesicles; K_{act}, the apparent dissociation constant for the interaction of T_α-GTPγS with PDE in the presence of a membrane; nK_{PL}, the apparent dissociation constant for the T_α-GTPγS–PDE activated complex and the phospholipid bilayer, defined as the product of the number of phospholipid molecules constituting an individual binding site (n) and the apparent dissociation constant for that site (K_{PL}); R, total photochemical products of bleached rhodopsin; ROS, rod outer segments; UW ROS, urea-washed rod outer segment membranes; GTPγS, guanosine 5′-[γ-thio]triphosphate; MOPS, 4-morpholinepropanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiotreitol; PMSF, phenylmethanesulfonyl fluoride.

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Miller et al. (1987), and Bennett and Clerc (1989)] or of positively charged detergents (Ho & Fung, 1984) or have resulted in only weak membrane enhancement of PDE activation (Phillips et al., 1989).

We have sought to determine whether the efficient activation of PDE by activated T_α observed in the presence of disk membranes can be obtained using protein-free bilayers of purified phospholipids and to characterize the requirements for efficient reconstitution. We report here a comparison of the ability of well-defined phosphatidylcholine vesicles and bovine rod outer segment membranes to serve as an activation surface, i.e., to bind activated T_α and PDE and to promote enhanced T_α-GTPγS activation of PDE.

MATERIALS AND METHODS

Reagents. GTPγS, MOPS, and DTT were obtained from Boehringer-Mannheim. EDTA was from Fisher Scientific. Reagents for electrophoresis were from Bio-Rad. Frozen dark-dissected bovine retinas were obtained from J. A. Lawson Packing (Lincoln, NE). Other reagents were obtained from the Sigma Chemical Co.

Buffers. The moderate-salt buffer contained 10 mM MOPS (pH 7.4), 30 mM NaCl, 60 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, and solid PMSF. The low-salt buffer contained 5 mM Tris (pH 7.4), 0.5 mM $MgCl_2$, 1 mM DTT, and solid PMSF. The pH assay buffer contained 20 mM MOPS (pH 8.0), 150 mM KCl, 2 mM $MgCl_2$, and 0.1 mM EDTA. Subunit separation buffer contained 10 mM MOPS (pH 7.4), 5 mM $MgCl_2$, 0.1 mM EDTA, and solid PMSF. All buffers were sterile filtered through 0.2-μm filters (Sartorius) immediately prior to use.

Rod Outer Segments and Protein Preparation. ROS were prepared under dim red light from frozen dark dissected bovine retinas as described (Fung & Stryer, 1980). ROS membranes stripped of peripheral proteins (UW ROS) were prepared by washing with 4 M urea (Yamanaka et al., 1985). These membranes were tested by papain treatment (Fung & Hubbell, 1978) to verify that >90% of R was accessible to the protease, and it is therefore assumed that approximately 50% of the total phospholipid was accessible to added proteins.

Preparation of transducin by GTP elution of hypotonically washed ROS and chromatography on hexyl-agarose was carried out as described (Fung et al., 1981). Activated transducin, T_α-GTPγS, was prepared either from holotransducin or by direct elution with GTPγS from low-salt-washed ROS. Activation of holotransducin (25 μM) was carried out in subunit separation buffer with 5 μM bleached rhodopsin in UW ROS and 10 μM GTPγS (3-mL total volume). The mixture was incubated at 4 °C for 2 h followed by removal of the membranes by centrifugation at 44 000 rpm in a Beckman TLA 100.3 rotor for 15 min at 4 °C. The volume of the supernatant containing the activated transducin was increased to 20 mL with subunit separation buffer and applied to a Reactive Blue 2–Sepharose CL-6B (Blue Sepharose) column (3 cm × 7 cm) equilibrated in the same buffer at 4 °C. The column was washed and the βγ subunit eluted in subunit separation buffer supplemented with 100 mM KCl (150 mL) followed by elution of the α subunit in the same buffer with 750 mM KCl (150 mL). Fractions were collected (5 mL) and analyzed by SDS–PAGE. The pooled proteins were concentrated by ultrafiltration under 50 psi N_2 with YM10 filters (Amicon) at 4 °C. All purified proteins were stored in 50% glycerol (v/v) at −20 °C. Alternatively, T_α-GTPγS was prepared by GTPγS (10 μM) extraction of washed ROS, followed by chromatography on Blue Sepharose as described above. T_α-GTPγS prepared on Blue Sepharose contained less than 1 PDE/3000 T_α-GTPγS. The T_α-GTPγS used in the direct-binding studies was prepared by mixing holotransducin with equimolar R (in urea-washed ROS membranes), in pH assay buffer, removing glycerol and unbound transducin by centrifugation, and eluting T_α-GTPγS and some $T_{\beta\gamma}$ from the membranes with pH assay buffer containing GTPγS. The protein was kept on ice and used within 24 h.

PDE was prepared essentially as described by Wensel and Stryer (1986, 1990). Bleached ROS membranes (300 retinas) were extracted with moderate- and then low-salt buffers. The low-salt extract was adjusted to 30 mM sodium phosphate (pH 7.2), 50 mM NaCl, and 25% (v/v) glycerol and applied to a 3 cm × 7 cm column of hydroxylapatite (Bio-Rad) equilibrated in the same buffer at 4 °C. The column was washed and the PDE was eluted with three stepwise increases in phosphate concentration: 80, 150, and 300 mM (2:1 Na^+ to K^+, pH 7.2) 25% in glycerol (v/v), 100–150 mL each.

PDE's membrane-binding domain was removed by incubating PDE wtih 0.01 mg/mL trypsin for 5 min (Figure 3A) or with 0.005 mg/mL trypsin for different times up to 20 min at room temperature. The reaction was stopped by adding soybean trypsin inhibitor to an inhibitor to trypsin ratio of 10 (w/w). Control samples were prepared by adding PDE to trypsin preincubated with inhibitor.

Phospholipid Vesicles. All vesicles were prepared with highly purified phospholipids. PC (egg yolk) was purchased from the Sigma Chemical Co. Large unilamellar vesicles (LUV) were prepared by modification of an extrusion method (Hope et al., 1985; Mayer et al., 1986). Organic solvent was removed from the phospholipid stock solution, 20–40 mg, under a gentle stream of dry N_2, and residual organic solvent was removed under reduced pressure for at least 2 h. The dried phospholipid film was resuspended in 1–2 mL of moderate-salt buffer with 0.1 mM EDTA, 1 mM DTT, and 0.05% NaN_3 (w/v) by vigorous agitation. The suspension was subjected to six freeze–thaw cycles in alternating dry ice–acetone and warm water baths and then forced, at least 10 times, through two stacked 0.1-μm polycarbonate filters (Costar/Nuclepore, Pleasanton, CA) encased in a 25-mm Millipore high-pressure stainless steel filter holder (XX45-025-00) coupled to a Waters HPLC pump with a 2-mL injection loop.

The size of vesicles prepared in this manner was determined by quasi-elastic light scattering using a Langley/Ford LSA2 light scattering spectrophotometer coupled to a Model 1096 correlator (Bloomfield & Lim, 1978). Diffusion constants were converted to hydrated radii by the Stokes–Einstein equation. The diameter of the PC LUV measured 103.8 ± 2.1 and 99.4 ± 2.2 nm, for two autocorrelation time constants which differed by a factor of 2, indicating a homogeneous size distribution.

LUV (100 nm) prepared using other extrusion devices after repeated freeze–thaw cycles have been shown to be predominantly unilamellar. That the vesicles made with our modified procedure were unilamellar was confirmed by ^{31}P NMR. The experiments were performed on a Bruker AM-400 (wide band) NMR spectrometer equipped with an Aspect-3000 data system operating at 161 MHz (^{31}P). The spectra, 8000 data points/scan with a spectral width of 16 kHz, were acquired in the unlocked mode with broad-band proton decoupling at 40 °C and externally referenced to 80% polyphosphoric acid. The ^{31}P spectrum of phosphate in phospholipid bilayers consists of a single resonance which can be split into two lines by addition of Pr^{3+} ions. One peak remains at the original

resonance and the other is paramagnetically shifted downfield, representing the phosphate inaccessible and accessible to Pr^{3+}, respectively (Brainard et al., 1980). The ratio of the integrated areas for these two lines was 0.99, indicating that about half of the phosphorus was accessible to the Pr^{3+} ions, i.e., located in the external leaflet, and half was not.

The multilamellar vesicles (MLV) used in binding experiments were prepared in an identical manner except that the aqueous suspension was forced through two 0.4-μm filters. Because of their large size, nearly all of such vesicles are multilamellar (Mayer et al., 1986). To ensure quantitative separation of PDE bound to the vesicles from PDE free in solution, the most rapidly sedimenting vesicles were selected by centrifugation for 10 min in a microcentrifuge ($\sim$13000g). The supernatant containing the smaller vesicles was removed, and the pellet was resuspended in a small amount of buffer (0.5–1 mL). The resuspended pellet fraction, designated 400-nm MLV, was the source of the phospholipid vesicles used in direct-binding experiments. The fraction of external phospholipid was determined with ^{31}P NMR as described above. For PC MLV the ratio of accessible to total phosphorus was 0.28. All phospholipid concentrations for PC vesicles and ROS membranes are expressed in terms of accessible phospholipid, as are apparent dissociation constants calculated from titration data.

PDE Assays. PDE activity was measured by continuously monitoring the amplified signal from a pH electrode (MI-410, Microelectrodes, Inc., Londonderry, NH) indicating the release of protons from cGMP (Liebman & Evanczuk, 1982), using 2 mM cGMP unless noted, at room temperature, in pH assay buffer with 0.1% ovalbumin (w/v) in a final volume of 200 μL. The measured hydrolysis rate was found to be linear with the concentration of active PDE throughout the range of total PDE activity used in these experiments. Reconstitution of T_α-GTPγS- and membrane-stimulated PDE activity was accomplished by adding the purified proteins and phospholipid vesicles or UW ROS membranes individually from concentrated stocks to the stirred solution. Total PDE activity in the sample was defined as the activity measured following addition of excess trypsin (typically, 0.25 mg/mL). PDE–membrane binding was directly determined in sedimentation experiments. PDE was mixed with the indicated amounts of membranes in the presence or absence of T_α-GTPγS (2 μM) in pH assay buffer to a final volume of 175 μL and a final glycerol concentration of 0.8% or less. PDE that bound the membranes was separated from free PDE by sedimentation in an airfuge, 28 psi ($\sim$130000g), for 20 min at room temperature. The amount of PDE present in the supernatant and pellet fractions was measured by activity following trypsin activation. GTPγS activation of PDE in native ROS membranes was determined by the pH method at room temperature. Sufficient GTPγS to give the indicated final concentrations was added to freshly bleached ROS membranes, 2 μM R, in pH assay buffer.

In order to estimate the uncertainty in activity measurements, we compared 23 independent measurements of trypsin-stimulated activity of PDE on 3 different days over a 7-day period. The activity for a nominal concentration of 10 nM PDE was found to be 39.5 μM cGMP hydrolyzed/s, with a standard deviation of $\pm$ 3.4 μM/s. In general, deviations among results from replicate samples measured on a given day were within this range. Larger deviations were occasionally observed when replicate results for multicomponent samples were obtained on different days; in some cases results from different days were averaged and their standard deviations plotted (e.g., in Figure 5D) to illustrate this day-to-day variation. However, no conclusions are drawn from such data sets unless supported by comparisons of data sets with small deviations (<10%) measured on individual days.

Electrophoresis. SDS–PAGE was performed essentially according to the method of Laemmli (1970). The proteins were precipitated with 5% (w/v) trichloroacetic acid before being solubilized in sample application buffer. Protein bands were visualized with Coomassie blue stain. Densitometric scanning of stained gels was performed using a Pharmacia LKB 2222-020 UltroScan XL laser densitometer.

Other Assays. Transducin and PDE concentrations were determined by the Coomassie blue-binding method (Bradford, 1976). The concentration of active PDE was determined by activity using a turnover number of 4200 cGMP s^{-1} (Hurley & Stryer, 1982) which was typically 0.4–0.6 of the value of the concentration determined by dye binding. Phospholipid concentration in UW ROS and vesicles was determined by analysis of inorganic phosphate (Chen et al., 1956). For the UW ROS used in these studies, the molar ratio, phospholipid/R was determined to be 60, based on the 500-nm absorbance of rhodopsin before bleaching.

Data Analysis. Maximal activity and apparent equilibrium dissociation constants (nK_{PL}) for the effect of membranes on T_α-GTPγS-mediated activation of PDE (Figure 2) were determined by nonlinear least-squares fitting of the data to hyperbolic binding curves for a single class of sites:

$$A = A_{max}L/(L + nK_{PL}) \quad (1)$$

where A is the PDE activity at each concentration of accessible phospholipid (L) and A_{max} is the PDE activity at saturating phospholipid. Both A and A_{max} are expressed as the percentage of total, trypsin-stimulated PDE activity present in each sample (see above, PDE Assays).

Two models for PDE–T_α-GTPγS interaction (Figure 5A,C,D) were considered: in one, a "low-affinity model", T_α-GTPγS is assumed to be homogeneous with regard to its ability to activate PDE and to activate it as a result of binding to relatively low affinity ($K_{act} > 10^{-7}$ M) binding sites. For this model, the PDE activity is predicted by eq 1 if C_T, the concentration of T_α-GTPγS, is substituted for L. Alternatively, the data were analyzed in terms of a "high-affinity model", in which only a small fraction of the total T_α-GTPγS is assumed to be capable of activating PDE, but is assumed to do so with relatively high affinity ($K_{act} < 10^{-9}$ M). For this model, the PDE activity, A, at each T_α-GTPγS concentration is predicted by eq 2. In this equation, f is the fraction of active

$$A = (A_{max}/S_t)\{-b - (b^2 - 4S_t f C_T)^{1/2}/2\} \quad (2)$$

T_α-GTPγS, C_T is the concentration of added T_α-GTPγS, and S_t is the total concentration of sites for T_α-GTPγS binding to PDE. S_t is assumed to be equal to twice the concentration of activatable PDE, which was determined from the PDE activity, A_{max}, observed at saturating C_T. The parameter b is defined as $b \equiv (-K_{act} - S_t - fC_T)$.

Values for f, A_{max}, and K_{act} were determined by a nonlinear least-squares fitting procedure. A first approximation for A_{max} was obtained from the mean activity at saturating C_T, and the first approximation of f was obtained by using $f = A_{max}S_t/C_T$ at the equivalence point. The equivalence point was estimated graphically as the intersection of two straight lines, one drawn through the ascending, linear portion of the data and the other drawn horizontal to the x-axis through the mean activity value at saturating C_T. K_{act} was then estimated by systematically varying its value until a minimum value of χ^2 was obtained.

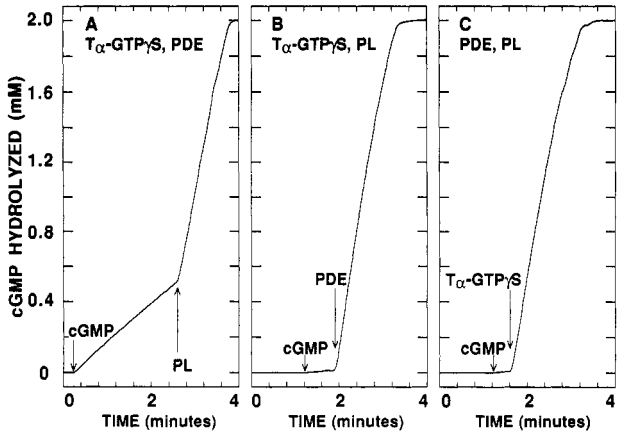


FIGURE 1: Reconstitution of T_α-GTPγS-stimulated PDE activity on phospholipid vesicles. cGMP hydrolysis resulting from the activation of 10 nM PDE by 0.6 μM T_α-GTPγS in the presence of 35 μM PC LUV was assayed by the pH method. The final components of each assay mixture were the same but the order of addition varied. (A) PC LUV were added to T_α-GTPγS and PDE. (B) PDE was added to T_α-GTPγS and PC LUV. (C) T_α-GTPγS was added to PC LUV and PDE. The T_α-GTPγS used in this experiment was the preparation of Figure 5A.

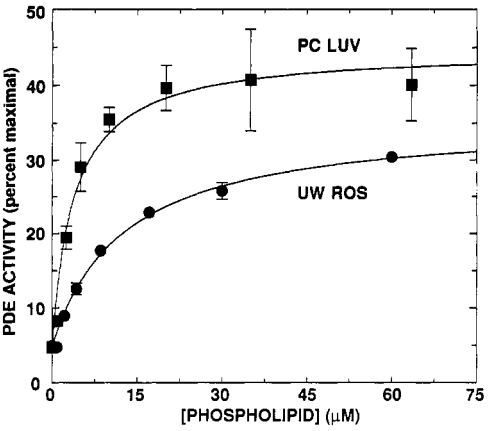


FIGURE 2: Phospholipid dependence of T_α-GTPγS-stimulated PDE activation. Titration of 10 nM PDE and 0.3 μM T_α-GTPγS with phospholipid membranes from PC LUV (closed squares) or UW ROS (closed circles). PDE activity was plotted as the percentage of the total trypsin-induced activity present in the sample (independent samples, mean ± SD, UW ROS, $n = 2$; PC LUV, $n = 3$). The curve was calculated from eq 1 using the values for nK_{PL} and A_{max} given in Table II. Activity present in the UW ROS (<1 active PDE/7900 R) was determined in control titrations, which contained 0.3 μM T_α-GTPγS but lacked added PDE, and subtracted from the total activity at each phospholipid concentration. The T_α-GTPγS used in this experiment was the preparation of Figure 5A.

Further minimization of χ^2 was obtained by an iterative procedure in which all three parameters (f, K_{act} and A_{max}) were varied until further iterations did not result in a lower value of χ^2. The final best fit values for f and A_{max} never varied from the original estimates by more than 2% of the final values. Thus, in this model f and A_{max} were very well determined by the data. The values reported in Table I are the best fit values $\pm\sigma_f$ or σ_A, calculated numerically as $\sigma_f = (2/\partial^2\chi^2/\partial f^2))^{1/2}$ or $\sigma_A = (2/(\partial^2\chi^2/\partial A_{max}^2))^{1/2}$ (Bevington, 1969). Because the value of K_{act} was much lower than the values of fC_T needed to elicit measurable PDE activation, the χ^2 surface is very asymmetric: χ^2 is very sensitive to increases in K_{act} above the best fit value but very insensitive to decreases in K_{act}. Therefore a meaningful value of σ could not be calculated, and only an upper limit could be reliably determined for K_{act}. The values reported in Table I are those giving the minimum χ^2 values, and the upper limit is taken as that value which resulted in a χ^2 value 50% higher than the minimum value.

Equation 2 was also used to analyze GTPγS activation of transducin and PDE in native ROS membranes (Figure 5B), with C_T replaced by C_G, the concentration of GTPγS, f replaced by 1, and S_t defined here as the total concentration of GTPγS-binding sites on transducin, determined by fitting, and $b \equiv (-K_{act} - S_t - C_G)$. The assumptions implicit in this treatment are that PDE in ROS is maximally activated (i.e., $A = A_{max}$) when all T_α has bound GTPγS, but that, again, only a fraction of the T_α-GTPγS formed [in this case given by $f = (2A_{max}/4200\ s^{-1})/S_t$] is competent to activate PDE, and that the dissociation constant for GTPγS binding to T_α is lower than K_{act}. Equations 1 and 2 are equivalent under conditions where the concentration of titrant, fC_T or fC_G, is much greater than S_t, the concentration of binding sites.

Direct measurements of PDE binding to membranes (Figure 6) were fit to theoretical curves with one binding constant (eq 1 with bound PDE substituted for A) or two binding constants:

$$B = \{B_{max1}L/(L + nK_{PL1})\} + \{(100 - B_{max1})L/(L + nK_{PL2})\} \quad (3)$$

where B represents the percentage of membrane-bound PDE at each phospholipid concentration (L). B_{max1} and nK_{PL} values were determined from the fit, with the constraint that $B_{max1} \leq 100\%$. Each value reported in Table II is the best fit value (± the estimated standard deviation) in the fit parameter calculated as described above for the parameters of the tight binding model.

RESULTS

T_α-GTPγS-Stimulated PDE Activity on Protein-Free Phospholipid Bilayers. The stimulation of PDE activity upon addition of PC LUV to a mixture of PDE and T_α-GTPγS shows that PC bilayers can substitute for native ROS membranes in supporting PDE activation by transducin (Figure 1). When cGMP was added to 10 nM PDE and 0.6 μM T_α-GTPγS in the absence of membranes, only a low level of PDE activity was observed, 9% of the trypsin-activated value. Addition of 35 μM PC LUV resulted in a large increase in activity to 55% of the maximal, trypsin-activated, level. Activation on the PC LUV was fast, occurring within the 1–4-s mixing time of the assay, and did not require preincubation of PDE and T_α-GTPγS with phospholipid or gradual increase in ionic strength (Figure 1A). Activation did not depend on preincubation of PDE with T_α-GTPγS; the increase in activity to 69% of the maximum was immediate even when PDE was added to T_α-GTPγS and phospholipid (Figure 1B). PDE was not activated by phospholipids alone, nor by proteases or surfaces in the assay environment. Activation of cGMP hydrolysis did not occur until T_α-GTPγS was added to the vesicles and PDE, activating PDE to 55% of its maximal activity (Figure 1C). This efficient activation of PDE by T_α-GTPγS on the surface of PC LUV indicates that a neutral protein-free bilayer is a sufficient activation surface and that rhodopsin or other integral membrane proteins are not required for activation.

The order in which the proteins and phospholipid were added to the assay mixture appeared to have a small but consistent effect on PDE activity. The highest activity was always obtained when PDE was the final component added. For example, PDE activity was slightly higher, 69% vs 55% of the trypsin-induced level, when added last (Figure 1, part B vs part A or C). This effect was even more pronounced in the

Table I: Fit Parameters for Two Models for T_α-GTPγS–PDE Interaction[a]

	high-affinity model		low-affinity model
	f	K_{act}[b] (nM)	K_{act} (nM)
T_α-GTPγS preparation 1[c]			
PC LUV	0.048 ± 0.005	0.04 (<0.25)	240 ± 28
T_α-GTPγS preparation 2[d]			
PC LUV	0.0048 ± 0.0005	2×10^{-7} (<0.036)	4300 ± 440
UW ROS	0.0056 ± 0.0007	0.01 (<0.025)	1100 ± 660

[a] Nonlinear least-squares fit of T_α-GTPγS titrations of PDE activity (Figure 5) to a high- (eq 2) or low- (eq 1) affinity model as described in Materials and Methods. [b] Best fit value, followed by the upper limit in parentheses, determined as described in Materials and Methods. [c] Preparation of Figure 5A. [d] Preparation of Figure 5C and D.

Table II: Fit Parameters for PDE–Membrane Interaction

	one binding constant (activ)[a]	
	A_{max} (%)	nK_{PL} (μM)
PC LUV	45 ± 2	3.8 ± 1
UW ROS	36 ± 1	13 ± 0.7

	one binding constant (bind)[b]		two binding constants (bind)[c]		
	B_{max} (%)	nK_{PL} (μM)	B_{max1} (%)	nK_{PL1} (μM)	nK_{PL2} (μM)
PC MLV					
$-T_\alpha$-GTPγS	59 ± 2	190 ± 11	40 ± 1	99 ± 6	3700 ± 270
$+T_\alpha$-GTPγS	58 ± 2	36 ± 4	28 ± 1	4.1 ± 0.4	500 ± 30
UW ROS					
$-T_\alpha$-GTPγS	37 ± 1	53 ± 5	32 ± 1	43 ± 4	7500 ± 2700
$+T_\alpha$-GTPγS	55 ± 2	65 ± 9	27 ± 2	9.6 ± 6	1000 ± 100

[a] Nonlinear least-squares fit of phospholipid titrations of PDE activity (Figure 2) with eq 1. [b] Nonlinear least-squares fit of phospholipid titrations of PDE binding (Figure 6) with eq 1. [c] Nonlinear least-squares fit of phospholipid titrations of PDE binding (Figure 6) with eq 3.

absence of ovalbumin, in which case the T_α-GTPγS- and phospholipid-induced PDE activity was more than 3-fold lower, 20% vs 69% of the trypsin-activated value, if PDE was the first component added rather than the last (data not shown). This behavior may be related to surface adsorption of PDE and/or the extreme sensitivity of PDE membrane-binding sites to proteolysis (Wensel & Stryer, 1986; Ong et al., 1989; Catty & Deterre, 1991), because assays in which the PDE was exposed to dilute conditions for the longest period of time in the absence of other proteins or phospholipids always resulted in the lowest T_α-GTPγS- and phospholipid-stimulated activity.

Ovalbumin, a hydrophilic protein, diminished these effects and was present in all assays reported here at a final concentration of 0.1%. Buzdygon and Liebman (1984) have shown that ovalbumin does not affect the light activation of PDE activity even at concentrations 10 times higher than those used in our studies. PDE activity induced by T_α-GTPγS or trypsin and PDE membrane binding in the presence of ovalbumin was at least 95% of the activity measured in the absence of ovalbumin. Ovalbumin however, significantly reduced PDE basal activity (6-fold, data not shown) probably by inhibition of surface activation and proteolysis. Two potent protease inhibitors from egg whites, ovomucoid and ovoinhibitor (Feeney et al., 1963) are likely contaminants of ovalbumin. We have found that water purified in a Milli-Q system can acquire, during a few days of storage, sufficient protease activity to eliminate PDE's membrane-binding ability in a few minutes. Autoclaving the water or filtering it through nitrocellulose (which binds proteins more efficiently than cellulose acetate) greatly reduced this activity.

Comparison of PC Bilayers and ROS as Enhancers of Activation. The phospholipid dependence of the activation reaction was examined by phospholipid titrations of 10 nM PDE in the presence of 0.3 μM T_α-GTPγS with either PC LUV or UW ROS at the membrane source (Figure 2). In the absence of phospholipid, PDE activity was low, approximately 5% of the maximum, and increased 6-fold and 9-fold at saturating phospholipid for UW ROS and PV LUV, respectively. Titrations consistently revealed two small but measurable differences between the abilities of the two types of membranes to enhance PDE activation: when UW ROS membranes were used, higher concentrations of phospholipid were required to reach maximal activity, and the maximal activity achieved at saturating phospholipid was lower than the maximal activity of PC LUV. Both titration curves could be fit to simple hyperbolic binding curves consistent with a single class of activating sites on PDE for each type of membrane (eq 1, solid lines in Figure 2). The apparent nK_{PL} derived from fitting the data for PC LUV was 3.8 μM, whereas a 3-fold higher value of 13 μM was observed for UW ROS (Table II) While the difference in maximal activity observed at saturating phospholipid with the two types of membranes was only 25% in the phospholipid titrations shown in Figure 2, greater activity on LUV was observed every time the two types were compared in a large number of experiments, including titrations comparing different compositions of phospholipid (unpublished observations). In some cases the activity induced by saturating LUV was more than 2-fold higher than the activity induced by saturating UW ROS in parallel experiments using identical preparations of T_α-GTPγS and PDE.

The observation of both lower affinity and lower maximal activation on ROS membranes suggest that these differences may not simply be due to differences in available phospholipid. The reason that ROS membranes are less effective could be the presence of rhodopsin at a density of $\sim 3 \times 10^4$ molecules/μm^2 in the rod disk membrane, which may inhibit, either sterically or through repulsive interactions, the protein–phospholipid interactions required for activation of PDE. At the experimental pH, T_α-GTPγS, PDE, and rhodopsin are all negatively charged. Structural features of the washed ROS may also contribute to their inefficiency. The membranes

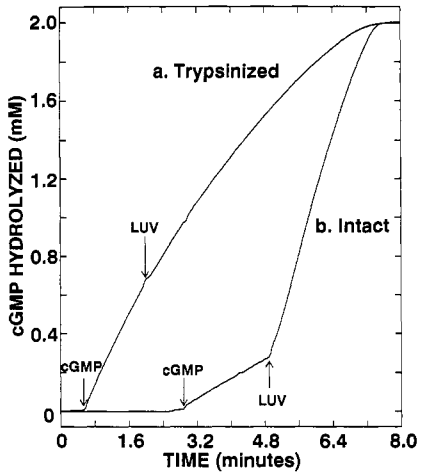


FIGURE 3: Effect of proteolysis on activation of PDE by PC LUV and T_α-GTPγS. cGMP hydrolysis resulting from T_α-GTPγS-mediated activation of partially trypsinized and intact PDE on PC LUV. (a) PC LUV (35 μM) added to trypsin-treated PDE (10 nM) and T_α-GTPγS (0.3 μM). The partial activation induced by the brief trypsin treatment (initial slope of trace a) was 16% of that induced by full trypsin activation. (b) PC LUV (35 μM) added to intact PDE (10 nM) and T_α-GTPγS (0.3 μM). The leftmost arrow in each trace indicates the addition of 2 mM cGMP. The T_α-GTPγS used in this experiment was the preparation of Figure 5A.

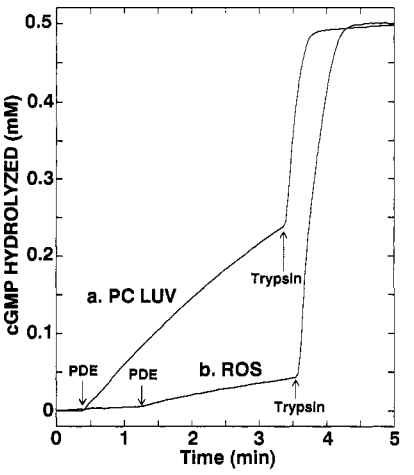


FIGURE 4: Effect of ionic strength on PDE activation. cGMP hydrolysis resulting from T_α-GTPγS-induced activation of PDE on UW ROS and PC LUV was measured in low-salt buffer [5 mM MOPS (pH 8) and 0.5 mM $MgCl_2$]. (a) PDE (10 nM) was added to T_α-GTPγS (0.3 μM), PC LUV (35 μM), and cGMP (0.5 mM). (b) PDE (10 nM) was added to T_α-GTPγS (0.3 μM), UW ROS (35 μM phospholipid), and cGMP (0.5 mM). The rightmost arrow in each trace indicates addition of trypsin (0.25 mg/mL). The T_α-GTPγS used in this experiment was the preparation of Figure 5A.

are expected to be very heterogenous in size and to include many small vesicles. We have observed that activation depends on vesicle size and that UW ROS are very similar to small unilamellar vesicles in their activation efficiency [Malinski and Wensel (1992), and unpublished observations].

Effect of Cleaving PDE's Membrane-Binding Domain on T_α-GTPγS-Mediated Activation. PDE is released from native ROS membranes by limited treatment with trypsin (Wensel & Stryer, 1986), which cleaves a short peptide from the C-terminus of both the α and β subunits (Ong et al., 1989; Catty & Deterre, 1991). When PDE was treated briefly with trypsin to remove its membrane-binding domain, while leaving 84% of its γ subunit intact, activation by T_α-GTPγS was no longer enhanced by PC LUV (Figure 3, trace a). However, addition of PC LUV to T_α-GTPγS and intact PDE, treated identically with inactivated trypsin (Figure 3, trace b), resulted in 6-fold enhancement of activity to 39% of the maximum. The PDE activity (16% of maximal) observed in the trypsin-treated sample before phospholipid addition (Figure 3, trace a) was due to partial loss of PDE_γ during trypsin digestion and was not dependent on T_α-GTPγS (data not shown). These findings suggest that the same regions of PDE responsible for binding PDE to ROS membranes are also required for enhancement of activity by phospholipid vesicles and T_α-GTPγS.

PDE Activation on PC LUV at Low Ionic Strength. In the isotonic environment of the rod cell, PDE is peripherally associated with the rod disk membrane. Decreasing the ionic strength of the solution elutes PDE from the membrane (Kühn, 1981; Baehr et al., 1979). Figure 4 shows the result of addition of PDE to T_α-GTPγS in the presence of either PC LUV or UW ROS in 5 mM MOPS (pH 8) and 0.5 mM $MgCl_2$. Activation on UW ROS (Figure 4, trace b) was very low, only 3% of the trypsin-activated value, consistent with very little PDE binding to the native membranes under conditions of low ionic strength. However, addition of PDE to T_α-GTPγS and PC LUV (trace a) resulted in activity that was 32% of the trypsin-stimulated maximum, and 12-fold greater than the activity observed in low salt with UW ROS. Thus the effects of low salt on PDE binding to ROS membranes and PDE activation by transducin appeared to be specific to the ROS membranes and not due to direct effects of low salt on PDE and transducin or to diminished affinity of PDE for phospholipids in low salt.

Transducin Dependence of Phospholipid-Mediated PDE Activation. Figure 5 shows the dependence of PDE activity on transducin concentration for two different preparations of T_α-GTPγS in the presence and absence of 35 μM phospholipid supplied by either PC LUV (Figure 5A and C) or UW ROS (Figure 5D) in the reconstituted system. The GTPγS dependence of PDE activity in native ROS membranes (Figure 5B) is shown for comparison. In the absence of membranes, T_α-GTPγS activated PDE much less efficiently, reaching only 9–12% of the total, trypsin-stimulated, activity at the highest T_α-GTPγS concentrations for either preparation (Figure 5A, C, and D; open symbols). However, when membranes were added to the same mixtures of T_α-GTPγS and PDE, the [T_α-GTPγS] dependence of PDE activation was very different for the two T_α-GTPγS preparations. A more potent T_α-GTPγS preparation (Figure 5A) consistently produced higher levels of PDE activity at lower T_α-GTPγS concentrations, reaching 38% of the maximal activity at 0.15 μM T_α-GTPγS and 69% of maximal at less than 0.5 μM T_α-GTPγS in the experiment shown. Activation of PDE by a less potent preparation of T_α-GTPγS was lower for both PC LUV (Figure 5C) and UW ROS (Figure 5D). Maximal T_α-GTPγS-stimulated PDE activity, 39% and 27% of the trypsin-activated value with PC LUV and UW ROS, respectively, required much higher concentrations of this T_α-GTPγS. The titrations shown in Figure 5A were performed with a different preparation of PDE than the titrations shown in Figure 5C and D, and the differences in maximal activity induced at saturating T_α-GTPγS probably reflect differences in the fraction of PDE that retained membrane-binding activity.

For each T_α-GTPγS preparation, the requirement for a large excess of T_α-GTPγS over PDE [[PDE] was 10 nM (Figure 5A) or 13 nM (Figure 5C and D)] could be due to a low-affinity interaction, with the K_{act} for the activation reaction given by the half-maximal point in the titration. Alternatively, it could be due to only a small fraction of T_α-GTPγS being competent to activate PDE, but doing so with

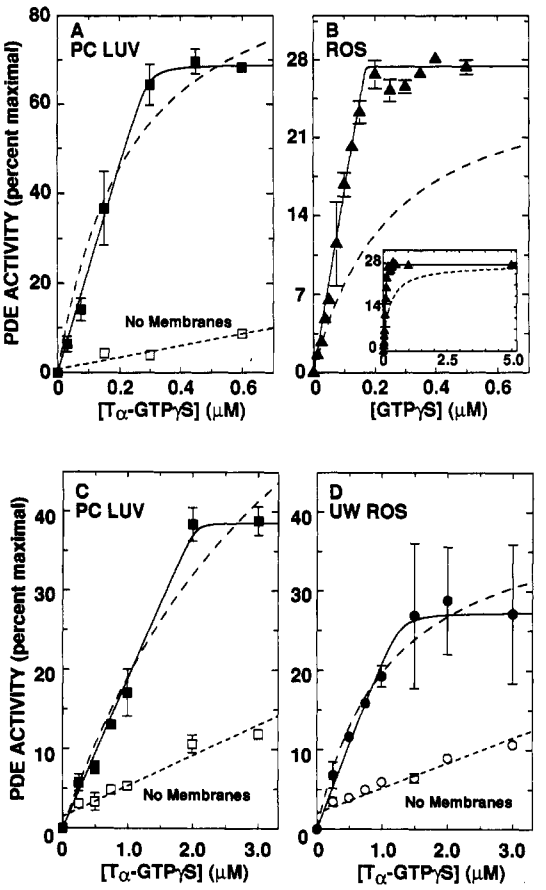


FIGURE 5: T_α-GTPγS dependence of PDE activation. PDE activity induced by T_α-GTPγS or GTPγS in the presence (closed symbols) and absence (open symbols; linear least-squares fit, short dashed lines) of membranes was measured by the pH method and plotted as the percentage of the total, trypsin-stimulated, activity in the assay (duplicate, independent samples, mean ±SD). All PDE activity was corrected for basal PDE activity, 0.5–2.7% of the total T_α-GTPγS- and membrane-dependent activity. (A) Activation of 10 nM PDE on 35 μM PC LUV. Theoretical curves were calculated for a low-affinity-binding model (eq 1, long dashed lines) and a high-affinity-binding model (eq 2, solid lines) using the parameter values given in Table I. (B) GTPγS stimulation of PDE activity in native ROS membranes (2 μM R). The theoretical curves were determined from eq 2 as described in the text. (C) Activation of 13 nM PDE on 35 μM PC LUV. PDE activity was plotted and theoretical curves were calculated as in (A). (D) Activation of 13 nM PDE on UW ROS (35 μM phospholipid) by T_α-GTPγS from the same preparation as in (C). PDE activity was plotted and theoretical curves were calculated as in (A).

relatively high affinity (K_{act} < 1 nM). In the former case the titration curves should be well represented by rectangular hyperolas (eq 1), while in the latter case a linear increase up to a plateau value is expected (eq 2). Titration data in several previous reports [e.g., Fung et al. (1981), Bennett (1982), and Tyminski and O'Brien (1984)] display the latter behavior, as do the results presented here. The solid lines in Figure 5 show the best fit curves obtained by assuming a small fraction of competent transducin activating with high affinity and the dashed lines (long dashes) show the fit obtained by assuming a low-affinity interaction between homogeneous T_α-GTPγS and PDE. The values obtained for the fit parameters are summarized in Table I. To account for the results using the low-affinity model, it was necessary to assume that the K_{act} had somehow increased 5–18-fold for the less active preparation. In the high-affinity model it was only necessary to assume that the fraction of active T_α varied in the two preparations. K_{act} values obtained from fitting the titration data (Figure 5A, C, and D) with eq 2 ranged from 0.0002 to 40 pM. Because all curves drawn for K_{act} values of <250 pM, using each set of f and A_{max} values given in Table I, are essentially indistinguishable, the results are consistent with an invariant, but low, value for K_{act}.

Activation of endogenous transducin and PDE in native ROS membranes by GTPγS (Figure 5B) also indicated a high-affinity interaction involving a small fraction of competent T_α. The theoretical curves (eq 2) were obtained using K_{act} values of 40 pM (solid line) and 240 nM (dashed line) derived from the fits of the data in Figure 5A. The total T_α capable of binding GTPγS was determined to be 170 nM (from the least-squares fit of the data in Figure 5B), in agreement with the value of 200 nM expected from the usual estimate of 1 T_α present/10 rhodopsin molecules in ROS. Assuming that all competent T_α is bound to PDE at saturating GTPγS, and that one fully active PDE requires two T_α molecules, competent T_α-GTPγS was determined to be 9 nM, or 5% of the total. This value is similar to the value of 4.8% determined for the fraction of competent T_α-GTPγS present in the purified protein preparation of Figure 5A.

Because the apparent K_{act} values estimated from the fits to eq 2 are much lower than the concentrations of PDE that could be conveniently assayed, they were not determined accurately from these titrations; however, the shapes of all four titration curves in Figure 5 are consistent with K_{act} < 250 pM and with K_{act} being the same for both preparations of T_α-GTPγS as well as for endogenous T_α in ROS membranes. For the low-affinity model (eq 1), much poorer fits (2–4-fold higher χ^2 values) were obtained, and the best fit K_{act} value differed greatly from one experiment to the next.

In all of the above activation titrations, including the GTPγS titration of transducin and PDE in ROS membranes (Figure 5B), the low K_{act} found for the T_α-GTPγS–PDE interaction also indicated that the dissociation constant for GTPγS binding to T_α may be significantly lower than the previously reported values of 50–100 nM (Kelleher, et al., 1986; Wessling-Resnick & Johnson, 1987). Determination of guanine nucleotide dissociation constants in the nanomolar and subnanomolar ranges by simple filter binding is not straightforward (Goody et al., 1991), and we are currently exploring alternative approaches for measuring T_α–nucleotide binding.

Enhancement of PDE Membrane Binding by T_α-GTPγS. The binding of PDE to UW ROS or vesicles was directly measured by determining the amount of trypsin-inducible PDE activity present in supernatants and pellets after sedimentation. As shown in Figure 6, PDE alone bound rather weakly to PC vesicles or ROS membranes, with somewhat stronger binding to UW ROS. The presence of 2 μM T_α-GTPγS increased the fraction of PDE found in the pellet fraction at all phospholipid concentrations tested, with this effect being somewhat more dramatic for PC MLV (Figure 6A) than for ROS membranes (Figure 6B).

PDE also enhanced T_α-GTPγS binding to membranes, as revealed by SDS–PAGE and densitometry of pellet and supernatant fractions (data not shown). In the absence of PDE, the amount of T_α-GTPγS in the phospholipid pellets was very low, but when both proteins were present with phospholipid vesicles, the amount of T_α-GTPγS in the pellet fraction was increased more than 5-fold to levels comparable to the amount of bound PDE.

Unlike the effect of increasing phospholipid concentration on PDE activation (Figure 2), which appeared to approach saturation at phospholipid concentrations below 100 μM, and could be easily fit to a single nK_{PL}, increased binding of PDE continued at near-millimolar concentrations of accessible

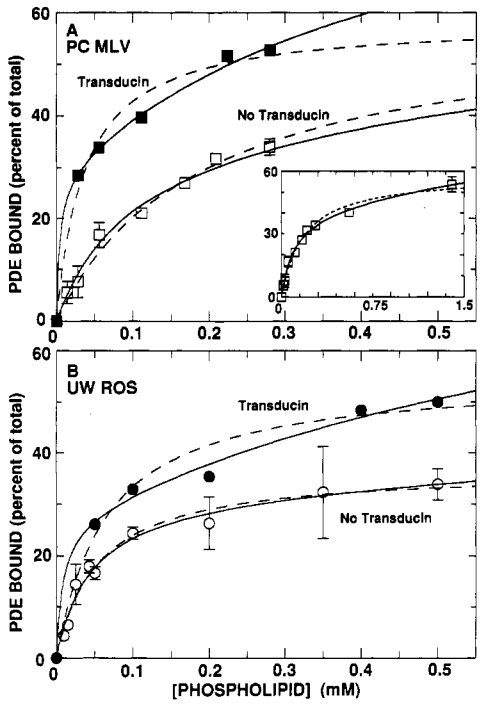


FIGURE 6: Phospholipid dependence of PDE binding. PDE bound to membranes was separated from free PDE by sedimentation. The activity in the supernatant and pellet fractions was measured by the pH method and percent bound PDE calculated according to $B = [PDE_p/(PDE_s + PDE_p)] \times 100$, where B is the percent PDE bound and PDE_p and PDE_s are the activities measured in the pellet and supernatant fractions, respectively (closed symbols, single measurement; open symbols, duplicate, independent samples; mean ± SD). Theoretical curves were calculated for one binding constant (eq 1, dashed lines) or two binding constants (eq 3, solid lines), using the parameter values given in Table II. (A) PDE (10 nM) bound to PC MLV in the presence (closed squares) and absence (open squares) of 2 μM T_α-GTPγS; (inset) phospholipid titration from (A) carried out to 1.5 mM phospholipid. (B) PDE (10 nM) bound to UW ROS in the presence (closed circles) and absence (open circles) of T_α-GTPγS (2 μM). PDE activity that remained in the tube (<3%) in the absence of membranes and activity that originated in the added UW ROS (<1 PDE/2000 R) were subtracted from the activity in the supernatants and pellets.

phospholipid (inset, Figure 6A). When the binding data in the presence of T_α-GTPγS were analyzed in terms of two populations of PDE with different affinities for the membranes (eq 3 and Table II), only the high-affinity component of binding to PC MLV and UW ROS appeared to be well correlated with activation. The calculated nK_{PL1} values of 4.0 μM for PC MLV and 9.6 μM for UW ROS are in reasonable agreement with the values of 3.8 μM for PC LUV and 13 μM for UW ROS determined from activity. A weaker binding component, with nK_{PL} values in the millimolar range, did not appear to be correlated with activation. While the binding data alone are not sufficient to distinguish between one and two types of binding interactions, the binding data together with the activation data support the conclusion that in addition to a fraction of PDE that interacts strongly with the membranes in a T_α-GTPγS-dependent way, there is a significant amount of PDE that displays only weak membrane binding and has lost its ability to be activated by T_α-GTPγS.

Effect of Trypsin Cleavage on PDE Binding to PC Bilayers. When PDE that had been treated with trypsin (0.005 mg/mL) for different lengths of time was mixed with PC MLV and sedimented, the amount of PDE present in the pellet decreased steadily during the digestion, with about half of the activity remaining in the pellet after 5 min (data not shown). Thus, removing the domain that binds PDE to ROS membranes not only removes its ability to be activated by transducin in a membrane-dependent manner (Figure 3) but also eliminates its ability to bind the PC vesicles. The extreme protease sensitivity of the membrane-binding domain may be the explanation for the finding that transducin-inducible PDE activity is almost always less than 100% of the trypsin-inducible activity and varies widely among PDE preparations.

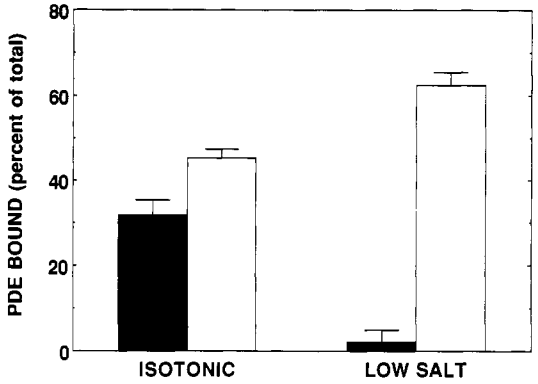


FIGURE 7: Effect of ionic strength on PDE binding to UW ROS and PC MLV. PDE (10 nM) and UW ROS (0.1 mM phospholipid, filled bars) or PC MLV (0.17 mM, open bars) were sedimented first in moderate-salt buffer and then resuspended in either low- or moderate-salt buffers. The activity remaining in the pellet after the second sedimentation was expressed as the percentage of activity in the pellet after the first sedimentation (duplicate, independent, assays; mean ± SD).

PDE Binding to PC MLV at Low Ionic Strength. In order to determine directly whether PDE could bind membranes in low-salt buffer, sedimentation experiments with either PC MLV or UW ROS were performed. The PDE–membrane mixture was sedimented first in moderate-salt buffer, and then the pellets containing membrane-bound PDE were resuspended in either moderate- or low-salt buffer and sedimented again. Figure 7 shows that while PDE bound both UW ROS and PC MLV in the moderate-salt buffer (32% and 45%, respectively), in low salt, 62% of the PDE remained bound to the PC MLV, while only 2% was found in the UW ROS pellets. This result confirms the conclusion from activation experiments (Figure 4) that PDE binds well to phospholipid in low salt.

DISCUSSION

The results presented here lead to the conclusion that protein–phospholipid interactions dominate the T_α- and membrane-dependent activation of PDE in ROS membranes. Rhodopsin and other proteins present in ROS membranes do not enhance these interactions and actually appear to interfere with them to some extent. Thus, bilayers of pure phospholipid represent an excellent model surface for studying transducin–PDE interactions.

Although T_α-GTPγS behaves in general as a freely soluble protein, its interactions with the PDE–membrane complex actually appear to be quite strong. Whether T_α-GTPγS effects on PDE activity are assayed in ROS membranes or in the reconstituted system, the shapes of the titration curves (Figure 5) imply a dissociation constant for the reaction of T_α-GTPγS with membrane-bound PDE of <250 pM.

To analyze the strength with which the membranes bind the protein complex(es), it is useful to estimate the size of a site comprising sufficient phospholipids to accommodate both PDE and T_α. An estimate for the minimum area required, calculated using the molecular weights and axial ratios of the proteins to determine cross-sectional areas for ellipsoidal

proteins, can be obtained for the "footprint" expected from a complex composed of one PDE and two T_α-GTPγS molecules. With axial ratios of 3:1 for T_α-GTPγS (oblate and prolate) and 9:1 and 12:1 for prolate and oblate PDE (Gillespie & Beavo, 1988), respectively, the minimum surface area required for one active complex would require 50–250 phospholipid molecules (70 Å^2/phospholipid), depending on whether the longest dimension of the complex is parallel or perpendicular to the plane of the membrane. If the ~4 μM nK_{PL} value for binding of the PDE–T_α-GTPγS complex to phospholipid (Figures 2 and 6A) is derived from sites consisting of 50–250 phospholipids (i.e., n = 50–250), then the dissociation constant for these sites would be (1.6–8) × 10^{-8} M, suggesting a fairly tight interaction.

A previous report using small unilamellar vesicles containing rhodopsin (Tyminski & O'Brien, 1984) estimated the number of accessible phospholipid molecules per PDE at saturating PDE to be 1500. This value of n is close to the value of 1690 phospholipid molecules estimated to be present per bound PDE when the concentration of phospholipid equals nK_{PL} under the conditions of Figures 2 and 6A. PDE titrations under conditions of limiting phospholipid and excess T_α-GTPγS are consistent with a value of n = 600–1600, suggesting K_{PL}'s in the range of 2.5–7 nM (unpublished observations). While it is unlikely that the activated complex can directly interact with this large number of phospholipid molecules, this number may reflect a maximum density at which the complexes can occupy the lipid surface before surface crowding and repulsive interactions between them become significant. In native ROS membranes, due to the high density of rhodopsin, unobstructed areas of phospholipid large enough to accommodate the activated complex may be a small fraction of the total surface area. In any case, it is clear that PDE can easily bind to membranes at much higher densities than that of 1 PDE/6000–7000 phospholipids found in native ROS [Liebman et al. (1987) and Wiedmann et al. (1988); see also Materials and Methods above].

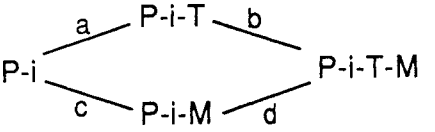
The data presented here display no indication of cooperativity in T_α's activation of PDE, nor do a number of previously published studies of PDE activation by transducin [e.g., Tyminski and O'Brien (1984), Fung et al. (1981), and Bennett (1982)]. However, this subject is still controversial, because there have been reports of a cooperative action of transducin linked either to the need for two transducins to bind a single PDE for significant activation (Bennett & Clerc, 1989) or to the need for two PDE_γ subunits to be removed from a single PDE for significant activation to occur (Whalen & Bitensky, 1989; Whalen et al., 1990). The linear shapes of the early portions of our T_α-GTPγS or GTPγS titration curves (Figure 5) argue strongly against both possibilities. Evidence against the latter possibility is also provided by the results of a previous study employing trypsin-activated PDE, in which PDE_γ inhibition of $PDE_{\alpha\beta}$ was found to be strictly linear with occupation of PDE_γ-binding sites (Wensel & Stryer, 1990).

Two possible sources of confusion in previous experiments that appeared to display cooperativity are as follows: (i) loss of activity at low protein concentrations due to surface adsorption or proteolysis and protection at higher protein concentrations. The presence of ovalbumin in our assays minimized such effects. (ii) the assumption that there is a homogeneous transducin population which binds PDE weakly rather than a heterogeneous population containing only a small fraction of competent transducin which binds PDE tightly.

How do phospholipid membranes achieve their dramatic enhancement effect? They do not do so by acting as containers for receptors that bind PDE and transducin. Specific receptors may be involved in localizing some peripheral membrane proteins, such as $p60^{src}$ (Resh, 1989; Resh & Ling, 1990), to specific membranes and may recognize lipids covalently attached to these proteins. However, if the isoprenyl groups on $PDE_{\alpha\beta}$ (Anant et al., 1992) or the N-linked myristic acid that has recently been found on T_α (Wensel & Yang, 1992) is important for these proteins to bind membranes, as has been suggested for PDE (Ong et al., 1989; Catty & Deterre, 1991; Qin et al., 1992), their contributions to membrane binding clearly do not require specific receptor proteins. Nor do membranes enhance activation by serving as protein concentrators. Such enhancement would require that both PDE and T_α-GTPγS independently concentrate at the membrane surface, while we find that in the absence of PDE, T_α-GTPγS has very low affinity for membranes.

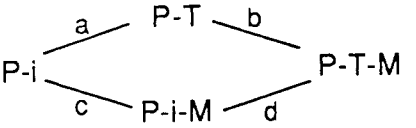
That the presence of T_α-GTPγS increases the amount of PDE bound to membranes and vice versa is the key to understanding the enhancement effect and indicates that the phospholipid-bound activated complex of T_α-GTPγS–PDE has a lower free energy than either an activated complex in solution or a noncomplexed state of T_α-GTPγS and PDE on the membrane surface. While the results presented here do not reveal the exact composition of this(these) complex(es), three different simple schemes can be proposed that are consistent with the observed results. These are presented as schemes I–III.

Scheme I



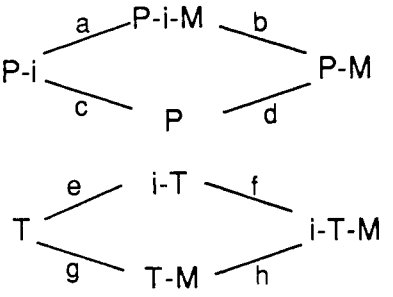
$$K_{Ia} > K_{Id}\ ;\ K_{Ic} > K_{Ib}$$

Scheme II



$$K_{IIa} > K_{IId}\ ;\ K_{IIc} > K_{IIb}$$

Scheme III



$$K_{IIIa} > K_{IIId}\ ;\ K_{IIIc} > K_{IIIb}\ ;K_{IIIe} > K_{IIIh}\ ;\ K_{IIIg} > K_{IIIf}$$

All equilibrium constants (K_{Ia}–K_{IIIh}) in the schemes are expressed as dissociation constants, except for the unitless constants characterizing the exchange reactions, $K_{IIa} \equiv$ [P–i][T]/[P–T][i] and $K_{IId} \equiv$ [P–i–M][T]/[P–T–M][i]. In all these reactions, P represents the PDE catalytic unit, $PDE_{\alpha\beta}$,

i represents PDE_γ, and T represents T_α-GTP or T_α-GTPγS. For simplicity, only one inhibitory γ subunit is represented for each P, which may be thought of as representing half of a functional $PDE_{\alpha\beta}$, with a single PDE_γ-binding site and half of the catalytic activity of $PDE_{\alpha\beta}$. The previous comparison of PDE activity with occupation of PDE_γ sites (Wensel & Stryer, 1990) supports the validity of this simplified treatment.

In Scheme I, a complex of T_α with holo-PDE binds membranes more tightly than does either holo-PDE alone or T_α alone (not shown). This is perhaps the simplest interpretation of our results, but it includes no role for the γ subunit of PDE (i), which has been shown to be capable of counteracting T_α-GTPγS activation of membrane-bound PDE (Wensel & Stryer, 1986, 1990). In Scheme II, T_α is shown displacing PDE_γ from the holoenzyme, leading to an enhanced affinity for the membranes; the membranes also enhance the ability of T_α to displace PDE_γ. In Scheme III, T_α forms a complex with PDE_γ (reactions IIIe and IIIh), driving the formation of PDE lacking one or both γ subunits, and this form binds membranes more tightly than does holo-PDE. To explain PDE's enhancement of T_α membrane binding in this scheme, it is also necessary to assume that the complex of T_α with PDE_γ binds membranes more tightly than does T_α alone.

Because the results employed here all used holo-PDE rather than individual subunits, they do not distinguish among these possibilities. However, the failure of PDE_γ either to bind membranes tightly in the absence of $PDE_{\alpha\beta}$ (Wensel & Stryer, 1990) or to be released from membranes when PDE is activated by transducin (Wensel & Stryer, 1986; Deterre et al., 1986) argues against Scheme II, which requires PDE_γ either to bind membranes by itself or to go into solution upon activation. Scheme III requires that T_α-GTPγS and PDE_γ form a fairly tightly membrane-associated complex. Although a complex of T_α-GTPγS–PDE_γ has been isolated in solution by ion-exchange chromatography (Deterre et al., 1986), and antibodies to T_α coimmunoprecipitate PDE_γ but not $PDE_{\alpha\beta}$ (Fung & Griswold-Prenner, 1989), a membrane-bound T_α-GTPγS–PDE_γ complex has not been demonstrated in the absence of $PDE_{\alpha\beta}$. Recent binding studies (Clerc & Bennett, 1992; Catty et al., 1992) with ROS membranes indicate that such a complex does not bind membranes. However, given our conclusions about the fraction of active T_α in typical preparations, it may be that formation of a membrane-bound T_α-GTPγS–PDE_γ complex can only be observed if a 20–500-fold molar excess of T_α is used. It is likely that the molecular interactions involved in activation are actually considerably more complicated than the simple schemes outlined above would suggest and that a mixture of various complexes between PDE subunits and T_α-GTP is present in the activated state.

Further studies with purified subunits will be required to resolve these issues, and reconstitution on LUV may represent one of the most straightforward approaches available. Binding assays that determine the effect of T_α-GTPγS on the binding of $PDE_{\alpha\beta}$ or PDE_γ and energy-transfer experiments that determine the proximity of individually labeled subunits or phospholipids to each other should be very helpful in clarifying the composition and stoichiometry of the membrane-bound complexes formed during PDE activation.

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Registry No. GTP, 86-01-1; cGMP phosphodiesterase, 9068-52-4.

CORRECTIONS

Involvement of Reactive Oxygen Intermediates in the Induction of c-jun Gene Transcription by Ionizing Radiation, by Rakesh Datta, Dennis E. Hallahan, Surender M. Kharbanda, Eric Rubin, Matthew L. Sherman, Eliezer Huberman, Ralph R. Weichselbaum, and Donald W. Kufe*, Volume 31, Number 35, September 8, 1992, pages 8300–8306.

Page 8303. Due to a printing error, the caption to Figure 5 is missing in the printed edition of the Journal. The figure and caption should appear as follows:

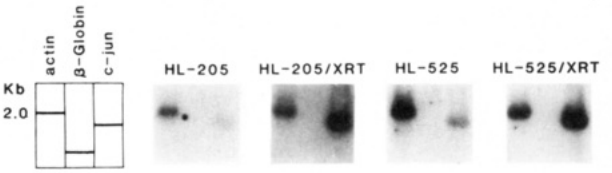

FIGURE 5: Transcriptional activation of the c-jun gene by ionizing radiation. HL-205 and HL-525 cells were exposed to 20-Gy ionizing radiation (XRT). After 2.5 h, nuclei were isolated, and newly elongated ^{32}P transcripts were isolated for hybridization to 2 μg of actin, β-globin, and c-jun DNA inserts after restriction enzyme digestion and Southern blotting. The solid lines in the schematic represent the positions of the DNA inserts.