Activation of cGMP Phosphodiesterase in Retinal Rods: Mechanism of Interaction with the GTP-Binding Protein (Transducin)

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ABSTRACT: The mechanism of activation of the cGMP phosphodiesterase by the GTP-binding protein in the disc membrane of retinal rods has been investigated by measuring the light-induced phosphodiesterase activity in reconstituted systems where the concentration of either the GTP-binding protein or the phosphodiesterase is varied. The results are consistent with the existence of two activator sites per phosphodiesterase functional unit: binding of one $G_{\alpha GTP}$ (α subunit of the G-protein with GTP bound) with high affinity (100 ± 50 nM) partially activates the enzyme ($V_{max1} \sim 0.05V_{max}$ to $0.10V_{max}$ of trypsin-activated phosphodiesterase); binding of a second $G_{\alpha GTP}$ with lower affinity (600 ± 100 nM) induces maximal activation ($V_{max2} \sim V_{max}$ of trypsin-activated phosphodiesterase). The two different states of activated phosphodiesterase have the same K_m for cGMP and the same pH dependence; they differ in their sensitivity to GMP. Micromolar concentration of protamines increases the affinity of the two activator sites and slightly increases V_{max1}. When G-protein is activated with GTPγS instead of GTP, the affinities of the two activator sites are not significantly modified, while V_{max1} appears to be increased.

Absorption of a photon by the photosensitive protein rhodopsin in retinal discs leads to the closure of sodium channels in the outer membrane of the rod cell [reviewed by Pugh and Cobbs (1986)]. The channels are kept open in the dark by direct action of cGMP molecules (Fesenko et al., 1985). Opening or closing of the channels is modulated by the concentration of cGMP, which is rapidly reduced upon illumination by a GTP-binding protein mediated activation of the rod cGMP phosphodiesterase (PDE).[1] Both the GTP-binding protein (G-protein or G, also called transducin) and the PDE are peripheral membrane proteins associated to the surface of the discs. The mechanism by which photoexcited rhodopsin activates the G-protein has been well studied. Interaction of excited rhodopsin with G catalyzes the exchange of GTP for bound GDP; this results in the dissociation of the α subunit from the remaining $\beta\gamma$ subunits, the α subunit of G with GTP bound ($G_{\alpha GTP}$) being the activator of the PDE (Fung & Stryer, 1980; Fung et al., 1981; Fung, 1983). The PDE is also composed of three subunits: two catalytic subunits P_α and P_β (88 and 84 kDa) and an inhibitory P_γ subunit (11 kDa) (Baehr et al., 1979; Hurley & Stryer, 1982). A recent report (Deterre et al., 1988) suggests that there are in fact two inhibitory P_γ subunits per $P_{\alpha\beta}$. Incubation of the PDE with trypsin degrades the P_γ subunit and at the same time activates the PDE, while addition of P_γ to trypsin-activated PDE inhibits the enzyme (Hurley & Stryer, 1982): by analogy, it has been proposed that light-induced activation of PDE is achieved by removal of the inhibitory subunit from the $P_{\alpha\beta}$ subunits by $G_{\alpha GTP}$ (Yamazaki et al., 1983); a complex of P_γ and $G_{\alpha GTP}$ has indeed been isolated (Deterre et al., 1986). The $G_{\alpha GTP}$-$P_{\alpha\beta\gamma}$ complex is however stable enough to be precipitated with anti-G_α monoclonal antibody (Navon & Fung, 1987) and to give rise to cross-linked products by $G_{\alpha GTP}$ or G_β and $P_{\alpha\beta}$ (Hingorani et al., 1988). Kinetic evidence moreover suggests that the active PDE is a complex with $G_{\alpha GTP}$ (Sitaramayya et al., 1986). It appears therefore probable that binding of $G_{\alpha GTP}$ to PDE activates the enzyme by modifying (rather than totally disrupting) the interaction between inhibitory and catalytic subunits.

Although it has been suggested from indirect evidence that the affinity of $G_{\alpha GTP}$ for the PDE is low (Liebman & Sitaramayya, 1984; Bennett, 1982), no direct measurement of the affinity has been published. Furthermore, the finding that PDE is regulated by two inhibitory subunits (Deterre et al., 1988) raises the question of the number of activator sites per PDE. A study of the equilibrium between $G_{\alpha GTP}$ and PDE, from measurements of PDE activity, is reported here.

Experimental Procedures

Bovine eyes were collected at a local slaughterhouse. Purified PDE, G-protein, and extensively washed "dark" or "bleached" membranes were prepared from purified rods (Kühn, 1985). Briefly, the rods are washed with isotonic buffer in order to remove all the soluble proteins. The membranes are then bleached in an ice bath and extracted in hypotonic buffer: the PDE [crude extract, containing about 9% $G_{\beta\gamma}$ (w/w) as the main contaminant] is released in the supernatant, while most of the G-protein remains attached to excited rhodopsin. Unless otherwise specified, the crude PDE extract is then purified from $G_{\beta\gamma}$ on a DE-52 Sephadex G-100 column (Baehr et al., 1979). After two additional hypotonic washes which remove the remaining PDE, the G-protein is extracted in hypotonic buffer with added GTP or GTPγS (75 μM). The "dark membranes" are prepared by washing the dark rod preparation at least five times with hypotonic buffer. "Bleached membrane" are obtained from the pellets used to extract PDE and G-protein. All experiments were carried out within 2 days following the extraction of the proteins, which were kept overnight in an ice bath.

Protein concentrations were measured by the method of Bradford (1976) using bovine serum albumin as standard.

PDE activity was measured with a pH meter according to Liebman and Evanczuk (1981); the experiments were carried

[1] Abbreviations: PDE, cGMP phosphodiesterase; P_γ, inhibitory subunit of PDE; $P_{\alpha\beta}$, catalytic subunits of PDE; R*, excited rhodopsin; G, GTP-binding protein or G-protein; G_α and $G_{\beta\gamma}$, subunits of G; $G_{\alpha GTP}$, α subunit of G with GTP bound.

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out in a thermostated pH vessel (20 °C), in 120 mM KCl, 1 mM $MgCl_2$, 1 mM MOPS, 170 μM GTP, and 500 μM cGMP, pH 7.0. Hydrolysis of all the cGMP added produced an acidification of about 0.2–0.3 pH unit. The concentration of G was always less than 25% that of rhodopsin, since the maximal capacity of the disc membranes for G was shown to be one per four rhodopsins (Baehr et al., 1982). The concentration of PDE was less than 8% that of rhodopsin. Under the ionic conditions used, G and PDE have a strong affinity (Kühn, 1985) for the membranes; with the above-cited proportions, all the PDE is assumed to reassociate to the membranes. PDE activity was initiated by a saturating flash which bleached 0.1% of the rhodopsin molecules (Bennett, 1982) or by addition of cGMP when bleached membranes or trypsin-activated PDE was used. PDE activation in the absence of G and membranes was induced by incubation with TPCK-trypsin (3 μg/pmol of PDE); the maximal activity was reached after 10–15-min incubation at 20 °C. Proteolysis was stopped by addition of soybean trypsin inhibitor (5 μg/μg of trypsin).

PDE activity was calculated in micromoles of cGMP per second from the initial slope and total amplitude of the pH change. In the experiments with [PDE] constant and varying [G], the turnover number was expressed in $cGMP \cdot s^{-1} \cdot (\text{total PDE})^{-1}$. In the experiments with [G] constant, when [PDE] ≫ [G], the maximum possible concentration of activated PDE is equal to the total concentration of G: the turnover number was then expressed in $cGMP \cdot s^{-1} \cdot (\text{total G})^{-1}$. The ratio of these values (V) to the trypsin-induced activity [V_{maxT}, measured in $cGMP \cdot s^{-1} \cdot (\text{total PDE})^{-1}$] gives the experimental V/V_{maxT} ratio.

Theoretical curves representing the variation of the activity as a function of total G-protein or PDE concentrations for different reaction schemes were calculated as described in the supplementary material (three models). The activity is proportional to the concentration of active PDE state(s): either $P_{\alpha\beta}$, $G\text{–}P_{\alpha\beta\gamma}$, or $G\text{–}P_{\alpha\beta}$ in model 1a, 1b, or 1c; G–PDE and G_2–PDE in model 2 (two active states); and G-PDE in model 3 (equivalent to model 1b in which dissociation of $P_{\alpha\beta}$ and P_γ is neglected). The theoretical V/V_{maxT} ratio is therefore equal to the [active PDE]/([PDE]$_{tot}$ or [G]$_{tot}$) ratio (models 1 and 3) or to ([G_2–PDE] + (V_{max1}/V_{max2})[G-PDE])/([PDE]$_{tot}$ or [G]$_{tot}$) (model 2).[2] The concentration of active PDE state(s) is expressed as a function of [PDE]$_{tot}$ and of the dissociation constant(s) according to the model. Under the conditions used in the experiments, the concentrations of free G or PDE are not known so that Hill or Scatchard plots cannot be used. Dissociation constants were adjusted by successive trials to obtain the best fit with experimental data.

Binding of labeled GTP (^{14}C or γ-^{32}P) and labeled GTPγS (^{35}S) was measured by filtration on Millipore filters (HAWP 02500, 0.45 μm). Washed membranes (bleached) and purified G-protein (separated from free nucleotides by gel filtration) were incubated for 30 s with labeled GTP or GTPγS and immediately filtered; blanks were obtained by filtrating the same volume of buffer with the same amount of membranes (no G-protein) for each GTP or GTPγS concentration. Concentrations of the cold GTP and GTPγS solutions used to dilute the labeled compounds were corrected for the amount of contaminant GDP, which was determined by FPLC filtration; it was found to be very large in GTPγS solutions.

Chemicals. GTP and GTPγS were purchased from Boehringer; labeled [γ-^{32}P]GTP and [^{14}C]GTP were from Amersham; [^{35}S]GTPγS was from NEN; protamines (Salmine grade X) were from Sigma.

Results

As previously reported, purified PDE can be activated in the absence of G-protein and membranes by incubation with trypsin, which degrades the inhibitory P_γ subunit (Hurley & Stryer, 1982). The maximal activity of the trypsin-activated enzyme under the experimental conditions used was between 700 and 1400 $cGMP \cdot s^{-1} \cdot PDE^{-1}$ in all experiments at 20 °C, pH 7; in a few experiments carried out at 35 °C, a turnover number of 4500–5500 cGMP/s (pH 7) was obtained. These values can be compared to previously reported turnover numbers of trypsin-induced PDE activity: 743–4044 cGMP/s (pH 8, 37 °C) (Sitaramayya et al., 1986), 2100 cGMP/s (pH 7.5, 37 °C) (Baehr et al., 1979), and 4000 cGMP/s (pH 8, 30 °C) (Hurley & Stryer, 1982). The trypsin-induced activity was assumed to correspond to full activation of all the PDE molecules.

PDE activity in solution before addition of trypsin is extremely low. It may correspond to the activity produced by dissociation of P_γ and $P_{\alpha\beta}$ subunits in the $P_{\alpha\beta\gamma}$ complex independently of activation according to the dissociation constant. It is thus possible to give an estimate for this constant from measurements of PDE activity before and after trypsin activation (see supplementary material). The upper limit deduced from several experiments is 10^{-13} M whether there is one or two P_γ subunits per PDE. It is comparable to the estimation of less than 10^{-11} M given in (Wensel & Stryer, 1986). The other values in the literature [15 nM in Sitaramayya et al. (1986) and 0.13 nM in Yamazaki et al. (1983)] were obtained by reassociating P_γ to trypsin-activated PDE, which can be significantly different from native $P_{\alpha\beta}$.

(1) Activation by G_{GTP}. PDE activity is measured at varying G concentrations ([PDE] constant: 30 and 80 nM) or at varying PDE concentrations ([G] constant: 30 and 80 nM). The ratio of these values to trypsin-induced activity of the same PDE preparation (V/V_{maxT}, see Experimental Procedures) is plotted in Figure 1. The PDE activity at varying G concentration for [G] > [PDE] reaches much higher values than the activity at varying PDE concentration for [PDE] > [G] (Figure 1A). On the contrary (Figure 1B), the activity at varying [PDE] for [PDE] < [G] is higher than the activity at varying [G] for [G] < [PDE]. Two characteristics are thus clearly apparent: the curve of PDE activity as a function of [G] has a sigmoidal shape, revealing a reduced activation at low G concentrations ([G] < [PDE]), while the curve obtained with varying PDE concentration is rapidly saturated, revealing a lower level of activation at high PDE concentrations (i.e., also when [G] < [PDE]).

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[2] The theoretical V/V_{maxT} ratio according to model 2 is

$$(V/V_{maxT})_{model2} = (V_1 + V_2)/V_{maxT}$$

with $V_{maxT} = V_{max}$ of trypsin-activated PDE, $V_1 = k_1$[G–PDE], and $V_2 = k_2$[G_2–PDE] (k_1 and k_2 being the rate constants for cGMP hydrolysis). V_1 and V_2 can be related to the V_{max} and concentration of each activated state:

V_1/V_{max1} =
[G–PDE]/{[PDE]$_{tot}$ (if [PDE] > [G]) or [G]$_{tot}$ (if [G] > [PDE])}

$$V_2/V_{max2} = [G_2\text{–PDE}]/([PDE]_{tot} \text{ or } [G]_{tot})$$

If $V_{max1}/V_{max2} = x$ and if $V_{max2} = V_{maxT}$, then

$$V_1/V_{maxT} = x[G\text{–PDE}]/([PDE]_{tot} \text{ or } [G]_{tot})$$

$$V_2/V_{maxT} = [G_2\text{–PDE}]/([PDE]_{tot} \text{ or } [G]_{tot})$$

Therefore

$$(V/V_{maxT})_{model2} = (x[G\text{–PDE}] + [G_2\text{–PDE}])/([PDE]_{tot} \text{ or } [G]_{tot})$$

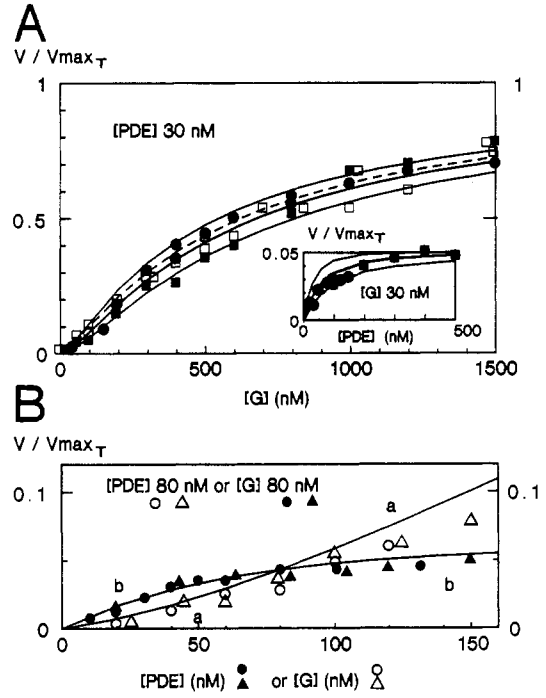

FIGURE 1: Variation of the PDE activity as a function of total G or PDE concentration. Symbols represent the experimental data [ratio of PDE activity (V) to V_{max} of trypsin-activated PDE (V_{maxT}), see Materials and Methods]. The curves drawn on the figure are the theoretical variations of V/V_{maxT} calculated according to model 2 (see Results and supplementary material). (A) PDE concentration 30 nM, varying G concentration. Inset: G concentration 30 nM, varying PDE concentration. (●) Crude PDE extract, dark membranes. Each point represents several measurements obtained with membrane concentrations differing by a factor 2–5 [with [rhodopsin] > 4[G] (Baehr et al., 1982)]; no significant difference was noted between these measurements. (■, □) Purified PDE, (■) dark membranes or (□) bleached membranes. [Rhodopsin] = 6[G] (for [G] > [PDE]) or 12[PDE] (for [PDE] > [G]). Calculated curves: variation of $([G_2\text{–PDE}] + (V_{max1}/V_{max2})[G\text{–PDE}]/[PDE]_{tot}$ ([PDE] = 30 nM) or $([G_2\text{–PDE}] + (V_{max1}/V_{max2}[G\text{–PDE}]/[G]_{tot}$ (inset, [G] = 30 nM). Solid lines: $V_{max1}/V_{max2} = 0.05$ [(top trace) $K_1 = 50$ nM, $K_2 = 500$ nM; (medium trace) $K_1 = 100$ nM, $K_2 = 600$ nM; (bottom trace) $K_1 = 150$ nM, $K_2 = 700$ nM]. Dashed line: $V_{max1}/V_{max2} = 0.10$ ($K_1 = 100$ nM, $K_2 = 600$ nM). (B) PDE concentration 80 nM, varying G concentration (filled symbols); G concentration 80 nM, varying PDE concentration (open symbols). (●, ○) Crude PDE extract; (▲, △) purified PDE. Calculated curves ($K_1 = 100$ nM, $K_2 = 600$ nM): (a) $V/V_{maxT} = ([G_2\text{–PDE}] + 0.05[G\text{–PDE}])/[PDE]_{tot}$; (b) $V/V_{maxT} = ([G_2\text{–PDE}] + 0.05[G\text{–PDE}])/[G]_{tot}$.

A possible cause for the observed reduced activation under conditions where [G] < [PDE] could be an inhibition by the P_γ subunit of the PDE itself if there is an excess of P_γ over $P_{\alpha\beta}$ (see supplementary material, Model 1: General Scheme of Interaction between $G_{\alpha GTP}$, $P_{\alpha\beta}$, and P_γ). The activity of the PDE decays more or less rapidly after its extraction from the membrane (according to the degree of purification, ionic conditions, and temperature) while heating does not destroy the inhibitory subunit, suggesting that the decay of the catalytic subunits is faster than that of the inhibitory subunit. This is likely to result in increasing the ratio of active P_γ over active $P_{\alpha\beta}$ as a function of the time after extraction from the membrane. In order to avoid any effect of a possible excess of P_γ subunit, experiments have been carried out with purified PDE within 2 days following extraction. When PDE was used prior to DE-52 Sephadex G-100 purification, experiments were carried out within 1 day. Identical results were obtained under these conditions with these two preparations (Figure 1).

The simplest model which is able to simulate the results if P_γ is not responsible for the inhibition observed when [G] < [PDE] involves two molecules of G (supplementary material, model 2): one which binds with higher affinity, inducing a low activation of the enzyme, and a second one which binds with lower affinity, inducing maximal activation. The active PDE is in that case a complex of PDE with either one or two G_{GTP} molecules:

model 2

$$G_{GTP} + PDE \underset{K_1}{\rightleftharpoons} G_{GTP}\text{–PDE} \quad (V_{max1}, \text{low activity})$$

$$G_{GTP} + G_{GTP}\text{–PDE} \underset{K_2}{\rightleftharpoons} (G_{GTP})_2\text{–PDE} \quad (V_{max2}, \text{high activity})$$

In this model, the G–PDE state is favored when [PDE] > [G], while the G_2–PDE state is favored when [PDE] < [G]. As a first approximation, we have assumed that the activity measured for [G] = 30 nM and [PDE] > 300 nM is only due to the G–PDE state, saturation corresponding to maximal concentration of complex (i.e., the concentration of the limiting component, which is G_{GTP} in this experiment). Supposing that $[G_{GTP}] = [G]_{tot}$, the activity can thus be expressed in cGMP hydrolyzed per second per total [G] (see Experimental Procedures): the V_{max} for G–PDE (V_{max1}) in Figure 1A (inset) calculated in this manner is 0.05 times the V_{max} of trypsin-activated PDE (V_{maxT}).

The formation of G_{GTP} has been shown to be much faster than GTP hydrolysis in rod fragments (Bennett & Dupont, 1985), suggesting that all the G-proteins should be in the activated G_{GTP} state as long as both R* and GTP are sufficient. It is possible however that, under the conditions used in the present experiments (reconstituted system), the G_{GTP}/G_{tot} ratio at the steady state is in fact lower than 1, i.e., that some of the G-proteins are in the inactive G_{GDP} or "empty" G states. In order to correct the value of V_{max1} for this possible error, we have measured the ratio of bound [γ-^{32}P]GTP to bound [^{14}C]GTP under conditions identical with those of Figure 1 ([G] constant: 30 or 80 nM): ^{14}C labeling corresponds to both G_{GTP} and G_{GDP} (produced upon hydrolysis of bound GTP), while ^{32}P labeling is only associated with G_{GTP}. The results (Table I) indicate that the $G_{GTP}/(G_{GTP} + G_{GDP})$ ratio is 66 ± 6% at low G concentration. Control experiments at higher G concentration (1 μM) give a higher value of 84 ± 5%. These values may be underestimated due to possible washout of cleaved γ-^{32}P during filtration (particularly at low G concentration, where much larger volumes of suspension are required in order to have a measurable amount of G on the filter compared to measurement at 1 μM G). Supposing that the amount of G without nucleotide bound is negligible, it follows

Table I: Measurement of the Proportion of Activated G $[G_{GTP}/(G_{GTP} + G_{GDP})]$ at Steady State from Binding of [γ-^{32}P]GTP and [^{14}C]GTP[a]

[G-protein]	bound [γ-^{32}P]GTP/bound [^{14}C]GTP (±SD)
30–80 nM	0.66 ± 0.06 (32 measurements)
1 μM	0.84 ± 0.05 (20 measurements)

[a] Binding of labeled GTP to G-protein was performed as described under Materials and Methods, under conditions identical with those used in the experiments of Figure 1 (three different G-protein preparations). The volumes of filtered suspension were 7 (30 nM G), 4 (80 nM G), and 0.5 mL (1 μM G); rhodopsin concentration was 6 times that of G. Results were independent of GTP concentration within the range studied (3–20 μM), consistent with previous measurements of GTP affinity [Bennett and Dupont (1985): K_D = 0.05–0.1 μM; Yamanaka et al. (1986): relative affinity of 0.5 μM]. The number of [^{14}C]GTP bound per G at saturation was between 0.77 and 0.97 independently of G concentration (27 measurements). If the concentration of G estimated by the method of Bradford is exact, the proportion of G with no nucleotide bound (or degraded) may therefore be between 3% and a maximum of 23%.

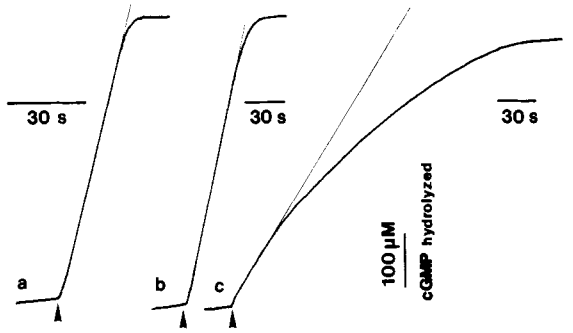

FIGURE 2: Recordings of pH variations associated with cGMP hydrolysis for (a) [PDE] = 30 nM, [G] = 1 μM; (b) [PDE] = 30 nM, [G] = 220 nM; and (c) [G] = 30 nM, [PDE] = 220 nM (G/PDE ratios: 33, 7, and 0.14, respectively). Dark membranes, activity initiated by a flash (arrow). Similar profiles are obtained when bleached membranes are used, and with GTP concentrations from 10 to 200 μM.

that the value of V_{max1}/V_{maxT} is probably between 0.05 ± 0.01 and 0.08 ± 0.01. Taking an absolute upper limit for "empty" or degraded G of 23% (Table I), the lower limit for G_{GTP}/G_{tot} is 50 ± 5%, which corresponds to a corrected V_{max1}/V_{max2} ratio of 0.10 ± 0.01.

The theoretical variation of the activity as a function of total PDE or G concentrations, $V/V_{maxT} = (x[G\text{–}PDE] + [G_2\text{–}PDE])/([PDE]_{tot}$ or $[G]_{tot})$,[2] is calculated as described under Experimental Procedures and in the supplementary material, taking $x = V_{max1}/V_{max2} = 0.05\text{–}0.10$ from Figure 1A, inset, and Table I. The best fit with the data in Figure 1 is obtained for $K_1 = 100 \pm 50$ nM and $K_2 = 600 \pm 100$ nM. With these values, the amount of G_2–PDE formed with [G] = 30 nM and [PDE] > 300 nM is less than 1% (Figure 4 of supplementary material), validating our estimate of V_{max1}.

(*2*) *Further Support for the Existence of Two Different States of Activated PDE.* Recordings of the pH variation associated with cGMP hydrolysis in two experiments with symmetrical concentrations of G and PDE (30 and 220 nM, corresponding to G/PDE ratios of 7 and 0.14) are reproduced in Figure 2b,c. These two recordings differ not only by the initial rate of hydrolysis (which is higher in the experiment where [G] > [PDE], as shown in Figure 1) but also by the variation of the reaction rate as a function of the remaining cGMP concentration: the rate of cGMP hydrolysis decays more rapidly in c than in b as the fraction of cGMP hydrolyzed increases. Similar hydrolysis profiles are observed whether PDE activation is induced by light through the transduction cascade or whether the reaction is initiated by addition of cGMP (the equilibrium between G_{GTP}, preactivated by use of bleached membranes, and PDE being reached before the onset of cGMP hydrolysis). The observation of different hydrolysis profiles under the two experimental conditions, which according to model 2 favor either the G–PDE or the G_2–PDE state, supports the existence of two different forms of activated PDE, having different V_{max} and differing by at least another property. We have therefore investigated the enzymatic properties of the two putative states of activated PDE (K_m for cGMP, pH dependence of the activity, and inhibition by GMP) which could account for these different rates of slowing down of cGMP hydrolysis.

The conditions chosen from model 2 under which most of the PDE activity is expected to be related to the G–PDE state or to the G_2–PDE state were respectively 30 nM G and 220 nM PDE (G/PDE = 0.14, Figure 2c) and 1000 nM G and 30 nM PDE (G/PDE = 33, Figure 2a). Under these conditions, the G–PDE/G_2–PDE ratio calculated according to model 2 (Figure 4 of supplementary material) is respectively ~65 and 0.63. In both cases, the pH dependence around pH 7 and the K_m for cGMP (75 ± 25 μM) are found to be identical, and similar to those of trypsin-activated enzyme (K_m = 60 ± 20 μM). The influence of GMP is however significantly different (Figure 5 of supplementary material): while a simple competitive inhibition between GMP and cGMP is observed for the G–PDE state, a combination of inhibitory and activatory effects which masks the inhibition at low cGMP concentrations is observed for G_2–PDE as well as for trypsin-activated PDE (not shown). This different sensitivity to GMP readily explains that the higher the G–PDE/G_2–PDE ratio, the faster the slowing down of the rate of cGMP hydrolysis.

(*3*) *Influence of the Concentration of Membranes on the Light-Induced PDE Activity.* If G_α is solubilized upon binding of GTP as suggested by centrifugation experiments (Kühn, 1985), modifying the amount of membranes should not modify the probability of interaction between $G_{\alpha GTP}$ and PDE. If on the other hand $G_{\alpha GTP}$ remains membrane bound as suggested by Liebman and Sitaramayya (1984), the variation of the PDE activity as a function of G concentration should be highly dependent on the concentration of membranes (increasing the membrane concentration by a factor k resulting in dividing the "real" $G_{\alpha GTP}$ and PDE concentrations by k). Several points in Figure 1, showing the variation of PDE activity as a function of G concentration, have been obtained with varying concentrations of membranes, ranging from a [rhodopsin]/[G] ratio of 4 to a ratio of 20: for a given G concentration, the activity of PDE is found to be totally independent of the amount of membranes. This result indicates that $G_{\alpha GTP}$ is soluble under our experimental conditions, either as a consequence of activation (Kühn, 1985) or due to the lowering of the affinity of G for the membrane by the purification procedure (Liebman & Sitaramayya, 1984), or both.

(*4*) *Activation by G_{GTP} in the Presence of Protamines.* It was previously reported that protamines activated purified solubilized PDE, saturation being obtained at a protamine concentration of 1 mg/mL (Miki et al., 1975). We find however that much lower concentrations of protamines markedly increase the light-induced PDE activity in the presence of G, GTP, and dark membranes (saturation of the effect: 20–30 μg/mL, corresponding to approximately 3 μM). At this low concentration, protamines are unable to activate purified PDE in solution, and no activation is observed in the dark in reconstituted systems, in agreement with Miki et al. (1975).

The variation of PDE activity as a function of G or PDE concentrations in the presence of 3 μM protamines (Figure 6 of supplementary material) is again consistent with two activator sites as proposed in model 2. It can be fitted with $K_1 = 0.1\text{–}5$ nM, $K_2 = 200\text{–}300$ nM, and V_{max1} (G–PDE) ~ $0.12\text{–}0.15V_{max2}$ (G_2–PDE) (without correction for the presence of inactive G). These results therefore suggest that at micromolar concentration protamines increase the affinity of the two activator sites for G_{GTP}, slightly increasing the V_{max} of the G–PDE state while that of the G_2–PDE state is not modified.

(*5*) *Activation by $G_{GTP\gamma S}$.* PDE activity is measured as a function of G-protein concentration ([PDE] constant) or as a function of PDE concentration ([G] constant) as in the experiments shown in Figure 1, except that G-protein is extracted with GTPγS.

The results obtained with $G_{GTP\gamma S}$ (Figure 3) are similar to those obtained with G_{GTP}, except that the activity of PDE with one activator bound (obtained from the saturation of the activity at high PDE concentrations in Figure 3B) is higher than

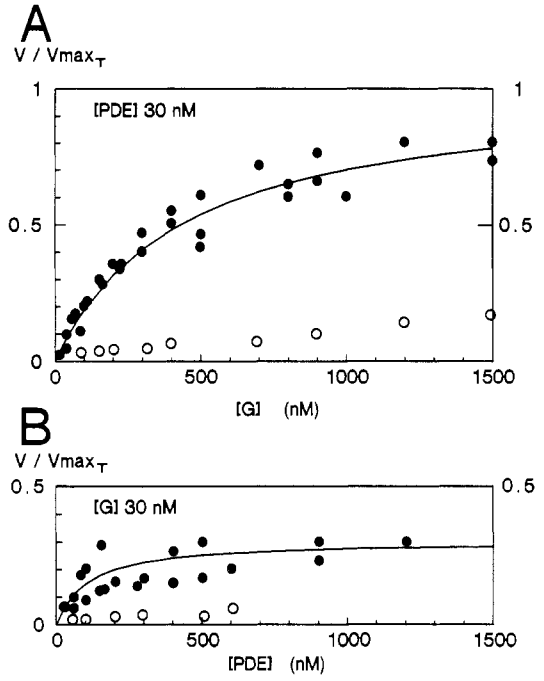

FIGURE 3: Activation of PDE by G preactivated with GTPγS: symbols represent experimental data (V/V_{maxT}, with $V_{maxT} = V_{max}$ of trypsin-activated PDE). (●) In the presence of membranes; (○) without membranes. Curves are theoretical curves calculated according to model 2 as in Figure 1 (see section 1 of Results and supplementary material), with the dissociation constants for the two activator sites which gave the best fit with the experimental data in Figure 1 (K_1 = 100 nM; K_2 = 600 nM) and with V_{max1}/V_{max2} = 0.3 (from Figure 3B, see text). (A) [PDE] = 30 nM; calculated curve V/V_{maxT} = ([G_2–PDE] + 0.3[G–PDE])/[PDE]$_{tot}$. Note that the theoretical curve does not appear sigmoidal as in Figure 1, due to the higher value of the V_{max1}/V_{max2} ratio. (B) [G] = 30 nM; calculated curve V/V_{maxT} = ([G_2–PDE] + 0.3[G–PDE])/[G]$_{tot}$.

that in the experiments with GTP. The theoretical curves drawn in Figure 3 were calculated with the dissociation constants obtained from Figure 1 (K_1 = 100 nM; K_2 = 600 nM), and by assuming $V_{max1} = 0.3V_{max2}$ (from Figure 3B).

Binding of [^{35}S]GTPγS to G-protein was measured by Millipore filtration under the same conditions and with the same G preparations as binding of [γ-^{32}P]GTP and [^{14}C]GTP (Table I). The affinity measured for GTPγS was K_D = 0.05 μM. The number of [^{35}S]GTPγS bound per G at saturation ([GTPγS] > 0.3 μM for 30 nM [G] or [GTPγS] > 2 μM for 1 μM [G]) was between 0.86 and 0.98 (13 measurements). This indicates that the proportion of "empty" G under these conditions, if any, is between 2% and a maximum of 14%; therefore, V_{max1}/V_{max2} may in fact be between 0.30 and 0.35.

The shapes of the pH traces obtained with G/PDE = 7 (activity mainly due to the G_2–PDE state according to model 2) and G/PDE = 0.14 (activity mainly due to the G–PDE state) in the presence of GTPγS (not shown) qualitatively resemble the shapes of the traces obtained under the same conditions in the presence of GTP (Figure 2), with faster decay of the rate of cGMP hydrolysis for G/PDE = 0.14.

When PDE is activated by $G_{GTP\gamma S}$ in the absence of membranes, the activity is very much reduced (Figure 3). It has been previously reported (Fung & Nash, 1983) that the activity induced by G_{GppNHp} in the absence of membranes is only 16% of the activity in the presence of membranes. The results in Figure 3 suggest that the reason for this reduced activity is at least in part due to a lowering of the affinity of PDE for G, though the V_{max} may also be slower as suggested by the results reported by Gillespie and Beavo (1988). Since $G_{\alpha GTP}$ is soluble under our conditions (Figure 1), this indicates that binding of PDE to the membrane facilitates its interaction with G.

Discussion

Our results support the existence of two distinct activated states of the PDE: a low-activity state, involving binding of one activator $G_{\alpha GTP}$, and a high-activity state, involving two $G_{\alpha GTP}$ per PDE. These results attractively match the recent proposal that PDE is regulated by two inhibitory subunits (Deterre et al., 1988), suggesting that the two active states are $P_{\alpha\beta}$ $(P_\gamma)_2$–G and $P_{\alpha\beta}$ $(P_\gamma$–$G)_2$. It should be noted that, although all calculations have been carried out by assuming a monomeric form of the enzyme, our results are also compatible with a model in which the PDE functional unit would be the dimer $(P_{\alpha\beta\gamma})_2$, the two activated states being in that case $(P_{\alpha\beta\gamma})_2$G and $(P_{\alpha\beta\gamma})_2G_2$.

The first $G_{\alpha GTP}$ binds with higher affinity (100 ± 50 nM) and the second $G_{\alpha GTP}$ with lower affinity (600 ± 100 nM). It is not known whether the two sites are different in the absence of activator or whether binding of the first $G_{\alpha GTP}$ to any of the two sites modifies the second site so that its affinity is reduced (negative cooperativity). The V_{max} of the first state is 5–10% that of the second. The affinities for the two activator sites are little or not modified when G_{GTP} is replaced by $G_{GTP\gamma S}$ (in the presence of membranes), while the V_{max} of the G–PDE state appears to be increased (30–35% of the V_{max} of the G_2–PDE state). This difference is perhaps related to the fact that in the presence of GTP some of the G-proteins could have GDP or no nucleotide bound; comparison of the number of [γ-^{32}P]GTP, [^{14}C]GTP, and [^{35}S]GTPγS bound per G under conditions identical with those used for measuring V_{max1} suggests however that the proportion of inactive G in the presence of GTP is not sufficient to account for the lower value of V_{max1}.

Although in model 2 active PDE is assumed to be a complex with the activator G_{GTP}, similar results would be obtained if the G–PDE complexes dissociate to G–P_γ and $P_{\alpha\beta}$ (fully or partially) as long as the dissociation of P_γ and $P_{\alpha\beta}$ subunits of PDE in the absence of activator is neglected:

$$G + P_{\alpha\beta\gamma_2} \underset{K_1}{\rightleftharpoons} (G\text{–}P_{\alpha\beta\gamma_2} \rightleftharpoons G\text{–}P_\gamma + P_{\alpha\beta\gamma}) \text{ (low activity)}$$

$$G + G\text{–}P_{\alpha\beta\gamma_2} \underset{K_2}{\rightleftharpoons} (G_2\text{–}P_{\alpha\beta\gamma_2} \rightleftharpoons 2G\text{–}P_\gamma + P_{\alpha\beta}) \text{ (high activity)}$$

This approximation seems to be justified by the very strong association between the subunits in the absence of activator [Wensel and Stryer (1986) and this paper]. The complete solution of a reaction scheme including two P_γ subunits (cf. model 1) having possibly different affinities for G_α subunits is extremely complicated and has not been attempted.

The activity of PDE from which only one of the two regulatory subunits has been removed by trypsin digestion was previously reported to be 50% of the activity of PDE with no γ (Deterre et al., 1988). The discrepancy with our results again suggests that trypsin-activated PDE is different from G_{GTP}-activated PDE (Navon & Fung, 1987; Hingorani et al., 1988; Sitaramayya et al., 1986); if the value of V_{max1} is indeed higher when PDE is activated by $G_{GTP\gamma S}$ instead of G_{GTP}, one of the possible explanations could therefore be a lesser stability of the G–PDE complex when GTP is replaced by GTPγS, leading to increased dissociation of $G_{\alpha GTP}$–P_γ from $P_{\alpha\beta}$.

In an attempt to understand the significance of the proposed mechanism of PDE activation for the cell function, we have calculated the theoretical variation of the proportion of G–PDE and of G_2–PDE (Figure 4A) and the variation of PDE activity

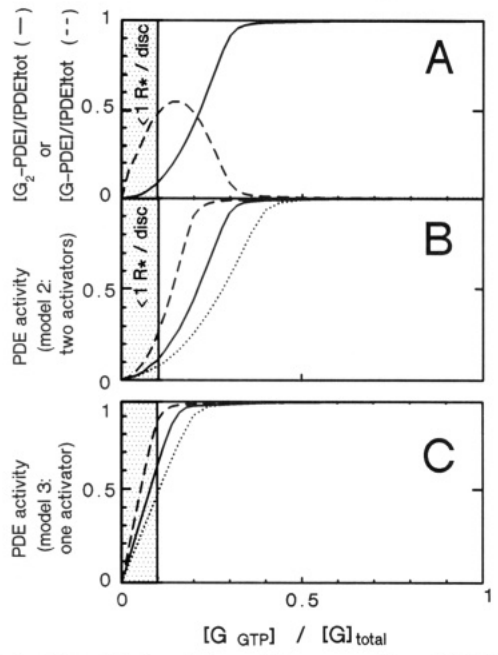

FIGURE 4: Calculated variation of the proportion of G–PDE and G_2–PDE complexes (A) and of PDE activity (B and C) in the rod as a function of the proportion of activated G-protein. (A and B) Calculations according to model 2 (see section 1 of Results and supplementary material), using the values for K_1 (0.1 μM) and K_2 (0.6 μM) which give the best fit with the experimental data shown in Figure 1. The estimated concentration of G in the rod is taken as 500 μM and that of PDE as 75 μM.[3] The variation of PDE activity $[V/V_{maxT} = ([G_2\text{–PDE}] + (V_{max1}/V_{max2})[\text{G–PDE}])/[\text{PDE}]_{tot}]$ for several PDE concentrations (50 μM, dashed line; 75 μM, solid line; 100 μM, dotted line) is shownn (B); it is not modified within the precision of the graph when V_{max1}/V_{max2} is varied from 0.05 to 0.10. (C) Theoretical variation of PDE activity for the same G and PDE concentrations as in (B), supposing that activation is produced by binding of only one G_{GTP} with $K = 0.6$ μM (model 3, Supplementary Material: $V/V_{maxT} = [\text{G–PDE}]/[\text{PDE}]_{tot}$). The dotted part of the diagrams corresponds to excitation of less than one rhodopsin molecule per disc according to previous estimates (Bennett & Dupont, 1985).

(Figure 4B) in the rod as a function of the percentage of activated G-protein (G_{GTP}/G_{tot}), using the affinities and the V_{max1}/V_{max2} ratio determined from Figure 1, Table I, and model 2. The total concentrations of G and PDE in the rod have been assumed to be 500 μM and 50–100 μM, respectively.[3] Maximal PDE activity is reached for 25–50% G-proteins activated, decreasing rapidly below. The existence of a first binding site for $G_{\alpha GTP}$ which induces a low activation shifts the response curve toward higher G_{GTP}/G_{tot} ratios as compared to the theoretical response curve calculated for only one activator site (model 3, supplementary material) having the same affinity as the second site of model 2 (Figure 4C): the response is thus attenuated below 10% activated G. We have previously estimated that activation of all the G-proteins requires excitation of 6–12 rhodopsins per disc (Bennett & Dupont, 1985) and therefore that excitation of one rhodopsin per disc activates about 10% of the G-proteins. The plot of Figure 4B is therefore consistent with the fact that the rod can respond to excitation of a single rhodopsin molecule; it suggests that all the PDEs in a rod should be activated by excitation of only two to five rhodopsin molecules per disc. If all the discs were equivalent, the probability that each of 100 (1000) discs has absorbed at least one or at least two photons would be 91% or 82%, respectively, when the rod absorbs 10^5 (10^7) photons. Although the actual number of discs required to saturate the response of the cell is unknown, this can be compared to the previous report (Penn & Hagins, 1972) that absorption of 10^5 photons per rod is required to almost saturate the rise time of the photoresponse.

The G–PDE state thus appears as a buffer pool, enabling the cell to cut off very low stimulations (such as spontaneous isomerization of rhodopsin) and to efficiently and rapidly adapt (increase or decrease) its reponse to very weak bleaches within the range from one to two to five excited rhodopsin molecules per disc. This process may in particular participate in the fast termination of the light response.

[3] If the diameter of a disc is 1 μm and if the proteins are concentrated in a band 60 Å wide on each side of the disc, the useful volume associated with one disc is approximately 10^{-17} L. Since one disc contains 3×10^4 molecules of rhodopsin, the concentration of rhodopsin in this "useful volume" is $(3 \times 10^4)/(6 \times 10^{23}) \times 10^{-17} = 5 \times 10^{-3}$ M. The concentration of G can therefore be estimated as 500 μM (10% of rhodopsin; Kühn, 1985) and that of PDE as 50–100 μM (1–2% of rhodopsin; Liebman & Sitaramayya, 1984).

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Supplementary Material Available

Calculation of the dissociation constant between the inhibitory P_γ and catalytic $P_{\alpha\beta}$ subunits of the PDE; (model 1) general scheme for the interaction between $G_{\alpha GTP}$, $P_{\alpha\beta}$, and P_γ (detailed calculations); (model 2) scheme of activation involving two activators $G_{\alpha GTP}$ per PDE, an approximation neglecting dissociation of $P_{\alpha\beta}$ and P_γ (detailed calculations); (model 3) simplified scheme of activation involving one G per PDE, neglecting dissociation of $P_{\alpha\beta}$ and P_γ; sensitivity of G–PDE and G_2–PDE to GMP; and activation of PDE by G_{GTP} in the presence of 3 μM protamines (9 pages). Ordering information is given on any current masthead page.

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Chemical Modification of Bovine Transducin: Probing the GTP-Binding Site with Affinity Analogues†

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ABSTRACT: The structure of the GTP-binding site of transducin, a signal-transducing G-protein involved in the visual excitation process, was studied by affinity labeling. Radioactive GTP analogues with reactive groups attached to different moieties of the GTP molecule were obtained and include 8-azido-GTP, P^3-(4-azidoanilino)-P^1-5′-GTP (AA-GTP), 5′-[*p*-(fluorosulfonyl)benzoyl]guanosine (FSBG), 3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}-GTP (ANPAP-GTP), the 2′,3′-dialdehyde derivative of GTP (oGTP), and a bifunctional cross-linking analogue, 8-azido-P^3-(4-azidoanilino)-P^1-5′-GTP (8-azido-AA-GTP). With the exception of FSBG, all of the analogues were found to bind to transducin specifically and serve as a cofactor to activate the retinal cGMP cascade or act as a competitive inhibitor for the GTPase activity of transducin. The labeling sites of these analogues were localized by tryptic peptide mapping. ANPAP-GTP and oGTP were unable to covalently modify transducin, suggesting that the 2′- and 3′-hydroxy groups on the ribose ring of GTP are not in direct contact with the protein. AA-GTP only labeled the T_α subunit of transducin and was localized on the 21-kDa tryptic fragment of T_α. This indicates that the phosphate moiety of the bound GTP is in direct contact with this peptide. On the other hand, 8-azido-GTP labeled both the T_α and $T_{\beta\gamma}$ subunits of transducin. The labeling on T_α was on the 12-kDa tryptic fragment, suggesting that the guanine ring binding site is composed of a different peptide fragment than the phosphate binding region. Treatment with the bifunctional analogue 8-azido-AA-GTP generated the cross-linked products of T_α and $T_{\beta\gamma}$. This observation implies that the guanine ring of the bound GTP on T_α could be in close proximity with $T_{\beta\gamma}$. The overall result of mapping the nucleotide binding site of transducin with affinity labeling is in complete agreement with the proposed model of T_α [Hingorani, V. N., & Ho, Y.-K. (1987) *FEBS Lett. 220*, 15-22] based on the crystal structure of the GTP-binding site of elongation factor Tu [Jurnak, F. (1985) *Science 230*, 32-36; la Cour, T. F. M., Nyborg, J., Thirup, S., & Clark, B. F. C. (1985) *EMBO J. 4*, 2385-2388].

Transducin (T),[1] a signal-transducing GTP-binding protein (Gilman, 1987; Stryer & Bourne, 1986), plays a pivotal role in the visual excitation process that involves a light-activated cGMP enzyme cascade [for reviews, see Liebman et al. (1987), Applebury and Hargrave (1986), Stryer (1986), and Chabre (1985)]. Transducin contains three polypeptide chains that can be functionally separated into two subunits, T_α (40 kDa) and $T_{\beta\gamma}$ (37 and 8 kDa) (Fung, 1983). In the rod outer segment (ROS) of the photoreceptor cell, photoexcited rhodopsin catalyzes the activation of hundreds of transducin molecules that via a GTP/GDP-exchange reaction lead to the formation of T_α–GTP complexes. The T_α–GTP complex dissociates from rhodopsin and in turn activates the latent cGMP phosphodiesterase (PDE), which rapidly hydrolyzes intracellular cGMP. The transient decrease in cGMP concentration causes closure of the cation channels on the plasma membrane and results in hyperpolarization of the rod photoreceptor cell (Fesenko et al., 1985; Yau & Nakatani, 1985). After the hydrolysis of the bound GTP, the transducin is deactivated and the T_α–GDP recombines with $T_{\beta\gamma}$ and can

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[1] Abbreviations: ROS, rod outer segment; T, transducin; T_α, α subunit of transducin; $T_{\beta\gamma}$, β and γ subunits of transducin; PDE, cyclic GMP phosphodiesterase; AA-GTP, P^3-(4-azidoanilino)-P^1-5′-GTP; oGTP, 2′,3′-dialdehyde derivative of GTP; ANPAP-GTP, 3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}-GTP; FSBG, 5′-[*p*-(fluorosulfonyl)-benzoyl]guanosine; Gpp(NH)p, guanosine 5′-(β,γ-imidotriphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); DTT, dithiothreitol; SDS, sodium dodecyl sulfate; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; kDa, kilodalton; EF-Tu, elongation factor Tu; TLC, thin-layer chromatography.

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