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Binding of cGMP to the transducin-activated cGMP phosphodiesterase, PDE6, initiates a large conformational change involved in its deactivation

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Summary

Retinal photoreceptor phosphodiesterase (PDE6), a key enzyme for phototransduction, consists of a catalytic subunit complex ($P\alpha\beta$) and two inhibitory subunits ($P\gamma$ s). $P\alpha\beta$ has two non-catalytic cGMP-binding sites. Here, using bovine PDE preparations, we show the role of these cGMP-binding sites in PDE regulation. $P\alpha\beta\gamma\gamma$ and its transducin-activated form, $P\alpha\beta\gamma$, contain two and one cGMP, respectively. Only $P\alpha\beta\gamma$ shows [^3H]cGMP binding with a $K_d \sim 50$ nM and $P\gamma$ inhibits the [^3H]cGMP binding. Binding of cGMP to $P\alpha\beta\gamma$ is suppressed during its formation, implying that cGMP binding is not involved in $P\alpha\beta\gamma\gamma$ activation. Once bound to $P\alpha\beta\gamma$ [^3H]cGMP is not dissociated even in the presence of a 1000-fold excess of unlabeled cGMP, binding of cGMP changes the apparent Stokes radius of $P\alpha\beta\gamma$, and the amount of [^3H]cGMP-bound $P\alpha\beta\gamma$ trapped by a filter is spontaneously increased during its incubation. These results suggest that $P\alpha\beta\gamma$ slowly changes its conformation after cGMP binding, *i.e.*, after formation of $P\alpha\beta\gamma$ containing two cGMPs. Binding of $P\gamma$ greatly shortens the time to detect the increase in the filter-trapped level of [^3H]cGMP-bound $P\alpha\beta\gamma$, but alters neither the level nor its Stokes radius. These results suggest that $P\gamma$ accelerates the conformational change, but does not add another change. These observations are consistent with the view that $P\alpha\beta\gamma$ changes its conformation during its deactivation and that the binding of cGMP and $P\gamma$ is crucial for this change. These observations also imply that $P\alpha\beta\gamma\gamma$ changes its conformation during its activation and that release of $P\gamma$ and cGMP is essential for this change.

Keywords

PDE; PDE6; cGMP binding; GAF domains; transducin; retinal phototransduction; G protein-mediated signal transduction; cGMP-binding-dependent protein conformational change

Introduction

Cyclic GMP phosphodiesterase (EC 3.1.4.17), classified as PDE6 in the PDE family, is one of the key enzymes for phototransduction in retinal photoreceptor OS. Its activation is G protein-mediated: illuminated rhodopsin stimulates GTP/GDP exchange on $T\alpha$ followed by dissociation of GTP- $T\alpha$ from $T\beta\gamma$. The GTP- $T\alpha$ activates PDE, resulting in a decrease in the cytoplasmic [cGMP], closure of cGMP-gated channels, and hyperpolarization of plasma membranes (1-3).

The inactive form of rod PDE is composed of a catalytic subunit complex, $P\alpha\beta$, and two inhibitory subunits, $P\gamma$ s, *i.e.*, $P\alpha\beta\gamma\gamma$ (4-10). A study using electron microscopy and image analysis of single particles (11) shows that bovine $P\alpha\beta\gamma\gamma$, $150 \times 108 \times 60 \text{ \AA}$, has the shape of a flattened bell with a handle-like protrusion ($\sim 30 \text{ \AA}$) and that the structure is divided into three distinct substructures by two holes. Except for the protrusion, the structure also appears to consist of two homologous structures arranged side by side. These characteristics are consistent with a model in which $P\alpha\beta\gamma\gamma$'s structure is determined by a dimer of homologous catalytic subunits consisting of two GAF regions and one catalytic region. Indeed, bovine $P\alpha\beta\gamma\gamma$ contains two cGMPs and these cGMPs bind tightly to substructures formed by GAF regions (12). These two substructures, called the non-catalytic cGMP-binding sites, are similar, but not identical, in shape and size (11). This implies that the manner of cGMP binding to each site and/or the role of cGMP binding to each site in PDE regulation, if present, may be different.

The current predominant model for PDE regulation is simple (13). For activation, GTP- $T\alpha$ interacts with $P\gamma$ in $P\alpha\beta\gamma\gamma$ and the GTP- $T\alpha$ - $P\alpha\beta\gamma\gamma$ complex, without altering the firm interaction between $P\alpha\beta$ and $P\gamma$, expresses a high cGMP-hydrolytic activity. For deactivation, GTP in the GTP- $T\alpha$ - $P\alpha\beta\gamma\gamma$ complex is hydrolyzed with help of RGS9 and accessory proteins, *i.e.*, the GTP is hydrolyzed after formation of a huge complex, and then $P\alpha\beta\gamma\gamma$ is recovered after dissociation of various proteins including GDP- $T\alpha$. This model conveniently explains rapid activation and deactivation of PDE; however, there is no clear evidence to show a firm and continuous interaction between GTP- $T\alpha$ and $P\alpha\beta\gamma\gamma$ during $P\alpha\beta\gamma\gamma$ activation as would be shown by the isolation of a complex of $P\alpha\beta\gamma\gamma$ with $T\alpha$ containing a hydrolysis-resistant GTP analogue such as GTP γ S. In addition, there is no definitive evidence to prove the formation of a GTP- $T\alpha$ - $P\alpha\beta\gamma\gamma$ complex containing RGS9 and accessory proteins and its decomposition during deactivation of GTP- $T\alpha$ -activated PDE.

Binding of cGMP to the non-catalytic site in $P\alpha\beta$ is believed to be involved in PDE regulation. Two models, the cGMP-regulated $P\alpha\beta$ - $P\gamma$ interaction model (14-18) and the cGMP-binding-direct regulation model (19), have been proposed to explain the role of cGMP-binding sites in PDE regulation. In the former model, the interaction between $P\alpha\beta$ and $P\gamma$ is dependent upon cGMP-presence at the non-catalytic site: When the non-catalytic sites of $P\alpha\beta\gamma\gamma$ are saturated with cGMP, GTP- $T\alpha$ activates $P\alpha\beta\gamma\gamma$ without changing the tight interaction between $P\alpha\beta$ and $P\gamma$, *i.e.*, a GTP- $T\alpha$ - $P\alpha\beta\gamma\gamma$ complex is formed and the complex expresses a high PDE activity. On the other hand, when the non-catalytic sites are not saturated, GTP- $T\alpha$ activates $P\alpha\beta\gamma\gamma$ through dissociation of $P\gamma$ complexed with GTP- $T\alpha$, *i.e.*, a $P\gamma$ -depleted PDE(s) is produced. $P\gamma$ in the GTP- $T\alpha$ complex enhances GTPase activity of $T\alpha$, the resulted GDP- $T\alpha$ instantly releases $P\gamma$, and the released $P\gamma$ deactivates the GTP- $T\alpha$ -activated PDE. In the latter model, binding of cGMP to the non-catalytic sites directly regulates PDE catalytic activity. These two models appear to explain some observations of cGMP binding to non-catalytic sites. However, as discussed later, these models have many ambiguous and controversial points. Thus, it is difficult to integrate these concepts smoothly into a coherent model for PDE regulation.

We have recently challenged the dominant model for PDE regulation by proposing a new and comprehensive model (11, 13, 20). In this model, GTP- α activates $\text{P}\alpha\beta\gamma\gamma$ by forming a complex with a $\text{P}\gamma$ thereby dissociating the $\text{P}\gamma$ -GTP- α complex. This occurs on membranes and is independent of the cytoplasmic [cGMP]. A significant portion of the $\text{P}\gamma$ -GTP- α complex is then released to the soluble fraction. Thus, $\text{P}\alpha\beta\gamma$ is the GTP- α -activated PDE. After hydrolysis of GTP, both soluble and membranous $\text{P}\gamma$ -GDP- α complexes deactivate $\text{P}\alpha\beta\gamma$ without liberating $\text{P}\gamma$. These $\text{P}\gamma$ -GDP- α complexes appear to have a preferential order in deactivating $\text{P}\alpha\beta\gamma$. This new model is based on the following observations: 1) $\text{P}\alpha\beta\gamma$, but not $\text{P}\alpha\beta$, is isolated only when OS homogenates are incubated with GTP γ S; 2) The ratio of $\text{P}\gamma$ / $\text{P}\alpha\beta$ in $\text{P}\alpha\beta\gamma\gamma$ and $\text{P}\alpha\beta\gamma$ is 2:1; 3) The enzymatic activity of $\text{P}\alpha\beta\gamma$ is ~12 times higher than that of $\text{P}\alpha\beta\gamma\gamma$ and inhibited by 30 nM $\text{P}\gamma$; 4) The basic structure of these PDE species is not changed when $\text{P}\alpha\beta\gamma\gamma$ is shifted to $\text{P}\alpha\beta\gamma$; 5) $\text{P}\gamma$ -GTP γ S- α is isolated from membranous and soluble fractions; 6) Both membranous and soluble $\text{P}\gamma$ -GDP- α complexes deactivate $\text{P}\alpha\beta\gamma$ without liberating $\text{P}\gamma$; 7) The membranous $\text{P}\gamma$ -GDP- α complex appears to be consumed earlier than the soluble $\text{P}\gamma$ -GDP- α complex; and 8) PDE regulatory mechanisms similar to this model are also found in mammalian and amphibian photoreceptors as well as in rods and cones. During these studies, we have also shown that 1) the interaction between $\text{P}\alpha\beta\gamma\gamma$ and GTP γ S- α is short-lived, indicating that GTP- α - $\text{P}\alpha\beta\gamma\gamma$ is an intermediate, but not the GTP- α -activated PDE; 2) free $\text{P}\gamma$ is not detected in any preparations, implying that $\text{P}\gamma$ always forms complexes with other proteins; 3) $\text{P}\alpha\beta\gamma\gamma\delta$ and $\text{P}\alpha\beta\gamma\delta\delta$ are formed when $\text{P}\alpha\beta\gamma\gamma$ and $\text{P}\alpha\beta\gamma$ are solubilized with $\text{P}\delta$, a prenyl-binding protein; 4) the stoichiometry of $\text{P}\alpha\beta\gamma\gamma\delta$ suggests that only one lipid-moiety may be involved in the interaction of $\text{P}\alpha\beta\gamma\gamma$ with membranes; and 5) the stoichiometry of $\text{P}\alpha\beta\gamma\delta\delta$ suggests that a lipid-moiety in $\text{P}\alpha\beta$ is also affected by $\text{P}\gamma$ dissociation.

In this study, we extend our model by integrating the role of cGMP binding to the non-catalytic site. We demonstrate that $\text{P}\alpha\beta\gamma\gamma$ and $\text{P}\alpha\beta\gamma$ contain two and one cGMP, respectively, that only $\text{P}\alpha\beta\gamma$ expresses [^3H]cGMP-binding activity and that $\text{P}\gamma$ inhibits [^3H]cGMP binding to $\text{P}\alpha\beta\gamma$. We also show that the cGMP binding to $\text{P}\alpha\beta\gamma$ is suppressed during $\text{P}\alpha\beta\gamma\gamma$ activation, *i.e.*, cGMP binding is not involved in $\text{P}\alpha\beta\gamma\gamma$ activation. We also suggest that cGMP binding to $\text{P}\alpha\beta\gamma$ slowly changes its conformation and that binding of $\text{P}\gamma$ accelerates the conformational change. Based on these studies, we propose that binding of cGMP to $\text{P}\alpha\beta\gamma$ is the first step in PDE deactivation.

Results

Binding of [^3H]cGMP to OS membranes

Bovine OS membranes contain a [^3H]cGMP-binding site(s) (Fig. 1A). Both GTP γ S-treated and non-treated membranes showed [^3H]cGMP-binding activities; however, the activity in GTP γ S-treated membranes was much higher than that in GTP γ S-non-treated membranes, indicating that GTP γ S- α somehow enhances the [^3H]cGMP-binding activity. On the contrary, the soluble fraction, whether obtained from GTP γ S-treated or non-treated OS homogenates, showed only a negligible [^3H]cGMP-binding activity (data not shown). This suggests that no protein in the soluble fraction contains the [^3H]cGMP-binding site and/or expresses the [^3H]cGMP-binding activity under our experimental conditions.

Solubilization and isolation of membranous proteins showed that a [^3H]cGMP-binding activity (Fig. 1B) was detected only in the fraction containing a protein-double (M_r ~88 kDa) (Fig. 1C) and that the activity appeared to be proportional to the level of the protein-doublet. These fractions also contained a PDE activity and the activity was proportional to the level of the protein-doublet (data not shown). The protein-doublet has been identified as $\text{P}\alpha\beta$ and 70-80% of $\text{P}\alpha\beta$ is extracted from membranes under these conditions (13, 20). These results suggest that the [^3H]cGMP-binding activity in membranes is due to a $\text{P}\alpha\beta$

complex(s). This implies that cone PDEs, $\text{P}\alpha'\alpha'/\text{P}\gamma'$ complexes, are also present and that a $\text{P}\alpha'\alpha'/\text{P}\gamma'$ complex(s) expresses a $[^3\text{H}]\text{cGMP}$ -binding activity. However, neither $\text{P}\alpha'$ nor its $[^3\text{H}]\text{cGMP}$ -binding activity could be identified. These failures, we believe, are due to its small abundance in OS. The soluble fraction also contained a $\text{P}\alpha\beta/\text{P}\gamma$ complex (peak **b** in Ref. 13); however, the complex showed only a negligible $[^3\text{H}]\text{cGMP}$ -binding activity (data not shown). This is consistent with the above-mentioned conclusion that $[^3\text{H}]\text{cGMP}$ -binding activity was not detected in the soluble fraction.

Interestingly, the $[^3\text{H}]\text{cGMP}$ -binding activity in $\text{GTP}\gamma\text{S}$ -treated PDE was higher than that in $\text{GTP}\gamma\text{S}$ -non-treated PDE (Fig. 1B). When OS homogenates are incubated with $\text{GTP}\gamma\text{S}$, the $\text{P}\alpha\beta$ content in membranes is increased 20-30% by binding of the $\text{P}\alpha\beta/\text{P}\gamma$ complex existing in the soluble fraction (13). Therefore, binding of the $\text{P}\alpha\beta/\text{P}\gamma$ complex to membranes and resulting expression of a $[^3\text{H}]\text{cGMP}$ -binding activity could increase the activity in membranes. However, the increase by $\text{GTP}\gamma\text{S}$ in the activity was much higher, ~ 2.4 times (Fig. 1B). In addition, $\text{P}\alpha\beta$ in the $\text{P}\alpha\beta/\text{P}\gamma$ complex has at most two cGMP-binding sites (12). Therefore, we conclude that even if the $\text{P}\alpha\beta/\text{P}\gamma$ complex could express a $[^3\text{H}]\text{cGMP}$ -binding activity, the greater part of the increase is due to an increase in the activity of a $\text{P}\alpha\beta/\text{P}\gamma$ complex(s) located on membranes. This is unexpected because previous studies using frog PDE/membranes (21, 22) showed that their $[^3\text{H}]\text{cGMP}$ -binding activity in GTP -non-treated PDE was much higher than that in GTP -treated PDE. We also note that this result, with the observation showing in Figure 1A, implies that $[^3\text{H}]\text{cGMP}$ binding to solubilized PDE species appears to be similar to that to membranous PDE species, *i.e.*, properties of cGMP binding to membranous PDE species may be estimated by studying cGMP binding to solubilized PDE species.

Identification of PDE species expressing $[^3\text{H}]\text{cGMP}$ -binding activity

$\text{GTP}\gamma\text{S}$ -non-treated membranes contain $\text{P}\alpha\beta\gamma\gamma$, and $\text{GTP}\gamma\text{S}$ -treated membranes have $\text{P}\alpha\beta\gamma\gamma$ and $\text{P}\alpha\beta\gamma$ as major species and a $\text{P}\alpha\beta/\text{P}\gamma$ complex as a minor species (20). These PDE species were extracted with a hypotonic buffer (Fig. 2A) or $\text{P}\delta$ in an isotonic buffer (Fig. 2C) and their $[^3\text{H}]\text{cGMP}$ -binding activities were measured after isolation. The use of $\text{P}\delta$ in an isotonic buffer may exclude a possible artifact(s) caused by the hypotonic extraction. OS homogenates were also treated with $\text{GTP}\gamma\text{S}$ in the presence of cGMP ($\text{GTP}\gamma\text{S} + \text{cGMP}$), and after isolation of $\text{P}\alpha\beta/\text{P}\gamma$ complexes, their $[^3\text{H}]\text{cGMP}$ -binding activities were measured (Fig. 2B). The result will be compared with the results shown in Figure 2A, as shown later.

1. $\text{P}\alpha\beta\gamma\gamma$ extracted by a hypotonic buffer. $\text{P}\alpha\beta\gamma\gamma$ was obtained from $\text{GTP}\gamma\text{S}$ -non-treated membranes (upper panel) and $\text{GTP}\gamma\text{S}$ -treated membranes (lower panel) (Fig. 2A). In the former preparation, the $[^3\text{H}]\text{cGMP}$ -binding activity appeared to be proportional to the level of $\text{P}\alpha\beta$, implying that the $\text{P}\alpha\beta\gamma\gamma$ may express a $[^3\text{H}]\text{cGMP}$ -binding activity. However, the molecular ratio of $[^3\text{H}]\text{cGMP}/\text{P}\alpha\beta$ was less than 0.01, indicating that only a negligible portion of the $\text{P}\alpha\beta\gamma\gamma$ expresses this activity. In the latter preparation, a small $[^3\text{H}]$ -radioactivity was detected in the fraction close to the $\text{P}\alpha\beta\gamma\gamma$ peak. However, the level of $[^3\text{H}]$ -radioactivity was not proportional to that of $\text{P}\alpha\beta$ in the $\text{P}\alpha\beta\gamma\gamma$ fraction, indicating that the $[^3\text{H}]$ -radioactivity is not attributable to $[^3\text{H}]\text{cGMP}$ bound to the $\text{P}\alpha\beta\gamma\gamma$, *i.e.*, the $\text{P}\alpha\beta\gamma\gamma$ does not show $[^3\text{H}]\text{cGMP}$ -binding activity and/or the $\text{P}\alpha\beta\gamma\gamma$, when it exists with $\text{GTP-T}\alpha$, appears to lose a portion that may express the $[^3\text{H}]\text{cGMP}$ -binding activity (upper panel).
2. $\text{P}\alpha\beta\gamma\gamma$ extracted with $\text{P}\delta$ in an isotonic buffer. The $\text{P}\alpha\beta\gamma\gamma\delta$ preparation was obtained from $\text{GTP}\gamma\text{S}$ -non-treated membranes (data not shown) and $\text{GTP}\gamma\text{S}$ -treated membranes (Fig. 2C). In the former preparation, the $[^3\text{H}]\text{cGMP}$ -binding activity appeared to be proportional to the level of $\text{P}\alpha\beta$; however, the molecular ratio of

$[^3\text{H}]\text{cGMP}/\text{Pa}\beta$ in the $\text{Pa}\beta\gamma\gamma\delta$ was less than 0.01. These observations are identical to those for $\text{Pa}\beta\gamma\gamma$ extracted with a hypotonic buffer (Fig. 2A, upper panel). In the latter preparation, $\text{Pa}\beta\gamma\gamma\delta$ appeared to show a small $[^3\text{H}]\text{cGMP}$ -binding activity (Fig. 2C, upper panel). However, its amount was not exactly proportional to the $\text{Pa}\beta$ level in the fraction, indicating that the $[^3\text{H}]$ -radioactivity was not due to $[^3\text{H}]\text{cGMP}$ bound to the $\text{Pa}\beta\gamma\gamma\delta$.

As shown later (Fig. 7), $\text{Pa}\beta\gamma\gamma$ can be trapped by the Millipore filter with a high efficiency, implying that the lack of $[^3\text{H}]\text{cGMP}$ -binding activity and/or the negligible level of $[^3\text{H}]\text{cGMP}$ -binding activity in $\text{Pa}\beta\gamma\gamma$ preparations are not due to the failure to trap the $[^3\text{H}]\text{cGMP}$ -bound $\text{Pa}\beta\gamma\gamma$. Taken together, our results strongly suggest that $\text{Pa}\beta\gamma\gamma$ does not express $[^3\text{H}]\text{cGMP}$ -binding activity and that negligible activities occasionally detected in fractions containing $\text{Pa}\beta\gamma\gamma$ may be artifacts caused by experimental procedures. The level of $[^3\text{H}]$ -radioactivity was not proportional to that of $\text{T}\alpha$ (Fig. 2C). This confirms that $\text{T}\alpha$ has no cGMP-binding site (23). The amino-acid sequence of $\text{T}\alpha$ also supports this notion. This is specifically noted here because we will use this information in a later discussion.

3. $\text{Pa}\beta\gamma$ and $\text{Pa}\beta/\text{P}\gamma$. Whether extracted with the hypotonic buffer (Fig. 2A, lower panel) or with $\text{P}\delta$ in the isotonic buffer (Fig. 2C), fractions containing these PDE species clearly showed $[^3\text{H}]\text{cGMP}$ -binding activities. In addition, the level of $\text{Pa}\beta$ was proportional to that of $[^3\text{H}]\text{cGMP}$ -binding activity in these fractions. These results indicate that both $\text{Pa}\beta\gamma$ and $\text{Pa}\beta/\text{P}\gamma$ express $[^3\text{H}]\text{cGMP}$ -binding activity.

We emphasize that $[^3\text{H}]\text{cGMP}$ -binding activity in the fraction containing $\text{Pa}\beta\gamma\delta\delta$ (Fig. 2C, upper panel) was similar to that in the fraction containing $\text{Pa}\beta\gamma$ (Fig. 2A, lower panel), although these activities were apparently different due to use of different amounts of OS homogenates and different volumes of the fraction for the assay. We confirmed this observation by comparing the $[^3\text{H}]\text{cGMP}$ -binding activity of $\text{Pa}\beta\gamma$ with that of $\text{Pa}\beta\gamma\delta\delta$ (data not shown). These results indicate that $\text{P}\delta$ binding to the lipid moiety of $\text{Pa}\beta$ does not affect the level of $[^3\text{H}]\text{cGMP}$ -binding activity in $\text{Pa}\beta\gamma$, implying that membrane binding of $\text{Pa}\beta\gamma$ may not affect its cGMP-binding activity. This implication also supports our above-mentioned view that properties of cGMP binding to membranous PDE species may be estimated by studying the cGMP binding to solubilized PDE species. We also note that the NaCl-gradient in the study shown in Figure 2C was modified to collect both rod and cone PDEs with fraction numbers similar to those for rod PDEs (Fig. 2A). Therefore, their elution profile was slightly different from that shown in Figure 2A. We have already shown that the elution profile of PDE species containing $\text{P}\delta$ is identical to that of PDE species without $\text{P}\delta$ when the same NaCl-gradient was used (20). Comparison of the $[^3\text{H}]\text{cGMP}$ -binding activity of cone PDE with that of $\text{Pa}\beta\gamma$ will be discussed later.

Contents of cGMP in $\text{Pa}\beta\gamma\gamma$ and $\text{Pa}\beta\gamma$

$\text{Pa}\beta\gamma\gamma$ and $\text{Pa}\beta\gamma$ were purified from GTP γ S-treated OS homogenates (Fig. 3A). These PDE species were clearly separated and characterization of these species including their specific activity and $\text{P}\gamma$ -sensitivity verified the clear separation (20). We also note that the level of protein staining with Coomassie blue is proportional to the M_r calculated based on its amino acid sequence under our staining conditions, *i.e.*, the $\text{P}\gamma/\text{Pa}\beta$ ratios also showed the clear separation (20). Molecular sieve chromatography of these PDE species also showed that the $\text{P}\gamma/\text{Pa}\beta$ ratio in these PDE species was not changed during their storage.

We found that 3.0 pmol of the $\text{Pa}\beta\gamma\gamma$ contained ~ 6.5 pmol of cGMP (Fig. 3B). This indicates that $\text{Pa}\beta\gamma\gamma$ contains 2 cGMPs. $\text{Pa}\beta\gamma\gamma$ isolated from GTP γ S-non-treated OS homogenates also contained 2 cGMPs (data not shown). These results indicate that non-catalytic sites of $\text{Pa}\beta\gamma\gamma$, whether located with or without GTP- $\text{T}\alpha$, are saturated by cGMP.

These results also suggest that the saturation is a reason for the lack of [^3H]cGMP-binding activity in $\text{Pa}\beta\gamma\gamma$. These $\text{Pa}\beta\gamma\gamma$ preparations had been exposed to cGMP-free conditions for at least one week. This suggests that these cGMPs bind tightly to $\text{Pa}\beta\gamma\gamma$, confirming a previous observation (12).

$\text{Pa}\beta\gamma$, 6.0 pmol, contained ~ 6.1 pmol of cGMP (Fig. 3B). This indicates that $\text{Pa}\beta\gamma$ contains one cGMP, *i.e.*, one of the non-catalytic sites in $\text{Pa}\beta\gamma$ is empty. The possibility that cGMP existing in $\text{Pa}\beta\gamma$ can be exchanged by [^3H]cGMP during the assay of [^3H]cGMP binding is quite low, as discussed later. Therefore, we conclude that the [^3H]cGMP-binding activity in $\text{Pa}\beta\gamma$ we observed is due to the binding of [^3H]cGMP to the empty site, *i.e.*, [^3H]cGMP-bound $\text{Pa}\beta\gamma$ contains one original cGMP and one [^3H]cGMP. These results also indicate that GTP- $\text{T}\alpha$ dissociates not only a single $\text{P}\gamma$ but also one cGMP from $\text{Pa}\beta\gamma\gamma$ during its activation. In other words, PDE activation is the mechanism to change $\text{Pa}\beta\gamma\gamma$ having two cGMPs to $\text{Pa}\beta\gamma$ having one cGMP, and PDE deactivation is the mechanism to shift $\text{Pa}\beta\gamma$ having one cGMP to $\text{Pa}\beta\gamma\gamma$ having two cGMPs. $\text{Pa}\beta/\text{P}\gamma$ (Fig. 2A lower panel and 2C upper panel) is a minor species that is difficult to purify (20). Therefore, the content of cGMP in $\text{Pa}\beta/\text{P}\gamma$ could not be measured.

$\text{Pa}\beta\gamma$ was exposed to cGMP-free conditions for more than 3 days. Under these conditions, the molecular ratio of cGMP/ $\text{Pa}\beta$ in $\text{Pa}\beta\gamma$ is always ~ 1.0 (Fig. 3B). This observation suggests that the affinity for cGMP is clearly different in $\text{Pa}\beta\gamma\gamma$'s two non-catalytic sites and that GTP γS - $\text{T}\alpha$ (GTP- $\text{T}\alpha$) releases cGMP only from the same one site in $\text{Pa}\beta\gamma\gamma$ during its activation. This also implies that GTP- $\text{T}\alpha$ dissociates $\text{P}\gamma$ from the same site in $\text{Pa}\beta\gamma\gamma$ during its activation.

Characterization of [^3H]cGMP binding to $\text{Pa}\beta\gamma$

Purified $\text{Pa}\beta\gamma$ showed a [^3H]cGMP-binding activity (Fig. 4A). The level of [^3H]cGMP binding reached a plateau as the [^3H]cGMP concentration increased. Scatchard plotting of this saturable [^3H]cGMP binding (Fig. 4A, Insert) indicates that $\text{Pa}\beta\gamma$ has one kind of cGMP-binding site with $K_d \sim 50$ nM. This is consistent with the above-mentioned view that [^3H]cGMP binds to the same site in $\text{Pa}\beta\gamma$. The level of bound [^3H]cGMP reached a plateau in less than 2 min under these conditions (Fig. 4B). Unlabeled cGMP, but not cAMP, competitively inhibited the [^3H]cGMP binding (Fig. 4C). This indicates that the [^3H]cGMP-binding site in $\text{Pa}\beta\gamma$ is cGMP-specific.

Trapping of [^3H]cGMP bound $\text{Pa}\beta\gamma$ to a Millipore filter

After incubation with [^3H]cGMP, $\text{Pa}\beta\gamma$ was applied to a molecular sieve column and the amount of [^3H]cGMP bound to $\text{Pa}\beta\gamma$ was calculated based on the [^3H]-radioactivity in the $\text{Pa}\beta\gamma$ fraction (Fig. 4D). We found that 70 μl of fraction 15, the peak fraction, contained 3.2 μg $\text{Pa}\beta\gamma$ (15.5 pmol) and 13.1 pmol of [^3H]cGMP, *i.e.*, $\sim 83\%$ of $\text{Pa}\beta\gamma$ in the fraction was occupied by [^3H]cGMP. The average level of the occupation was $\sim 86\%$ in three experiments. These results indicate that $\sim 100\%$ of $\text{Pa}\beta\gamma$ binds [^3H]cGMP under these conditions. The result will also be confirmed later (Fig. 5). However, only $\sim 17\%$ of the activity was detected when 70 μl of the fraction was applied to the filter and the [^3H]cGMP-binding activity was obtained based on the [^3H]-radioactivity trapped by the filter (Fig. 4D and its insert). This shows that the Millipore filter traps $\sim 17\%$ of the [^3H]cGMP-bound $\text{Pa}\beta\gamma$ existing in the assay mixture. We could not get the result showing that 100% of $\text{Pa}\beta\gamma$ expressed the [^3H]cGMP-binding activity. We believe that this is due to an artifact caused by our experimental procedures, because the $\text{Pa}\beta\gamma$ preparation we obtained appears to contain one kind of $\text{Pa}\beta\gamma$ (20) and [^3H]cGMP, once bound to $\text{Pa}\beta\gamma$, is not dissociated even in the presence of a 1000-fold excess of unlabeled cGMP (Fig. 5). In Figure 4D, fraction 15 apparently showed that 100% of $\text{Pa}\beta\gamma$ binds [^3H]cGMP. This is due to our intention to show

the ratio of [^3H]cGMP-binding activity measured by the filter. By the way, ~18.2% of the [^3H]cGMP-bound $\text{Pa}\beta\gamma$ in the assay mixture was trapped by the filter in the result shown as Figure 4A. We emphasize that this low rate does not affect the properties shown in Figure 4A, 4B and 4C, because these properties are not affected by the low efficiency of the filter to trap [^3H]cGMP-bound $\text{Pa}\beta\gamma$.

GTP γ S-Ta-activated and P δ -extracted cone PDE, $\text{Pa}'\alpha'\gamma'\delta\delta$ (20), expressed a [^3H]cGMP-binding activity (Fig. 2C, upper panel). We note that all $\text{Pa}'\alpha'\gamma'\gamma'$ complexes present were activated to $\text{Pa}'\alpha'\gamma'$ under our conditions (20). Interestingly, the level of [^3H]cGMP-binding activity in fraction 13 was ~5 times higher than that of fraction 27 (Fig. 2C, upper panel). A similar observation was also obtained when these PDEs were extracted with a hypotonic buffer (data not shown). These results indicate that ~85% of [^3H]cGMP-bound $\text{Pa}'\alpha'\gamma'$ was trapped by the Millipore filter under the following assumptions: (a) Contents of $\text{Pa}\beta$ and $\text{Pa}'\alpha'$ in these fractions are similar, (b) [^3H]cGMP binds to all $\text{Pa}'\alpha'\gamma'$ complexes, (c) $\text{Pa}'\alpha'\gamma'$ has one cGMP binding site, (d) P δ binding does not affect the level of [^3H]cGMP-binding activity in $\text{Pa}'\alpha'\gamma'$, and (e) ~17% of [^3H]cGMP-bound $\text{Pa}\beta\gamma$ is trapped by the Millipore filter. We note that the level of protein staining with Coomassie blue is proportional to the M_r calculated based on its amino acid sequence under our staining conditions (20). Thus, amounts of $\text{Pa}\beta$ and $\text{Pa}'\alpha'$ can be compared by comparing their staining levels in the same gel. We found that the stained level of $\text{Pa}\beta$ was similar to that of $\text{Pa}'\alpha'$ (Fig. 2C, lower panel). This indicates that levels of $\text{Pa}\beta$ and $\text{Pa}'\alpha'$ are similar, *i.e.*, assumption a was proven. As described, we found that the [^3H]cGMP-binding activity of $\text{Pa}'\alpha'\gamma'\delta\delta$ was similar to that of $\text{Pa}'\alpha'\gamma'$, *i.e.*, assumption d was proven. Assumption e was also proven, as described above. Assumptions b and c are not yet proven; however, these assumptions are reasonable if characteristics of the [^3H]cGMP binding to $\text{Pa}\beta\gamma$ are taken into consideration. Therefore, we conclude that the low trapping rate is specific to [^3H]cGMP-bound $\text{Pa}\beta\gamma$.

Conformational change of $\text{Pa}\beta\gamma$ by cGMP binding

After incubation with [^3H]cGMP for 30-min (*i.e.*, after binding of [^3H]cGMP to ~100% of $\text{Pa}\beta\gamma$), dissociation of [^3H]cGMP bound to $\text{Pa}\beta\gamma$ was followed with or without 1 mM unlabeled cGMP (Fig. 5A). We found that the level of [^3H]cGMP binding to $\text{Pa}\beta\gamma$ was not changed even in the presence of 1 mM unlabeled cGMP at least for the first 5 min. Under the similar conditions, [^3H]cGMP binding to $\text{Pa}\beta\gamma$ reached the maximum in less than 2 min (Fig. 4B), indicating that the 5-min incubation was enough to chase [^3H]cGMP bound to $\text{Pa}\beta\gamma$ if indeed the [^3H]cGMP could be chased. Therefore, this observation indicates that [^3H]cGMP, once bound to $\text{Pa}\beta\gamma$, cannot be dissociated. This strongly suggests that $\text{Pa}\beta\gamma$, after binding of [^3H]cGMP, changes its conformation, especially the conformation of its non-catalytic site and/or a region(s) near the non-catalytic site, and that the $\text{Pa}\beta\gamma$, after changing its conformation, firmly holds the [^3H]cGMP. A large conformational change initiated by cGMP binding has been reported in one of GAF domains in cone PDE (24). A similar conformational change may occur when [^3H]cGMP binds to $\text{Pa}\beta\gamma$, *i.e.*, when $\text{Pa}\beta\gamma$ having one cGMP is shifted to $\text{Pa}\beta\gamma$ having two cGMPs.

To further prove that binding of cGMP changes $\text{Pa}\beta\gamma$'s conformation, we directly compared the relative compactness (Stokes radius) of cGMP-treated $\text{Pa}\beta\gamma$ with that of cGMP-non-treated $\text{Pa}\beta\gamma$ (Fig. 5B). This method has been used to show a conformational change by cGMP binding in PDE5 (25-27). After incubation of $\text{Pa}\beta\gamma$ with or without cGMP for 30 min on ice, these $\text{Pa}\beta\gamma$ s were applied to a gel filtration column and PDE activity was measured to identify the fraction containing $\text{Pa}\beta\gamma$. The cGMP-non-treated $\text{Pa}\beta\gamma$, as expected, was eluted as a single peak with the peak activity in fraction 38. The [^3H]cGMP-binding activity was also observed in these fractions. However, the cGMP-treated $\text{Pa}\beta\gamma$ was eluted as two peaks, the major peak in fraction 34 and the minor peak in fraction 38, and only the $\text{Pa}\beta\gamma$ in fraction 38 showed the [^3H]cGMP-binding activity. These observations indicate that the

apparent Stokes radius of cGMP-treated $\text{Pa}\beta\gamma$ was 4-7 Å larger than that of cGMP-non-treated $\text{Pa}\beta\gamma$, *i.e.*, the Stokes radius of $\text{Pa}\beta\gamma$ appears to be increased when $\text{Pa}\beta\gamma$ having one cGMP is shifted to $\text{Pa}\beta\gamma$ having two cGMPs. We note that the difference in the Stokes radius was observed in the Tris buffer; however, the difference was less clear in a phosphate buffer (data not shown). This may be due to a tendency of $\text{Pa}\beta\gamma$ to change its structure in a Tris buffer (11). We also note that 50 μl of the peak fraction of the cGMP-non-treated $\text{Pa}\beta\gamma$ contained 2.4 μg $\text{Pa}\beta\gamma$ (11.6 pmol of $\text{Pa}\beta\gamma$) and bound 9.90 pmol [^3H]cGMP. This indicates that ~85% of the $\text{Pa}\beta\gamma$ expressed [^3H]cGMP-binding activity, confirming that almost all $\text{Pa}\beta\gamma$ complexes show the [^3H]cGMP-binding activity (Fig. 4D). We also note that the major peak of the cGMP-treated $\text{Pa}\beta\gamma$ showed no ability to bind [^3H]cGMP, confirming that cGMP, once bound to $\text{Pa}\beta\gamma$, is not dissociated (Fig. 5A).

Rate of the conformational change in $\text{Pa}\beta\gamma$

The level of [^3H]cGMP-binding abruptly increased after a 10-min incubation (Fig. 5A). The level was increased ~3 times the level at time 0 after 20-min (Fig. 5A) and ~4 times after 40-min (data not shown). Since ~100% of $\text{Pa}\beta\gamma$ present bound [^3H]cGMP during the pre-incubation, these observations indicate that the amount of [^3H]cGMP-bound $\text{Pa}\beta\gamma$ trapped by the filter abruptly increased.

The incubation of [^3H]cGMP-bound $\text{Pa}\beta\gamma$ was initiated by addition of unlabeled cGMP or water (Fig. 5A). The increase in the trapped level of [^3H]cGMP-bound $\text{Pa}\beta\gamma$ was observed after addition of 1 mM unlabeled cGMP, indicating that the increase is not due to new binding of [^3H]cGMP to $\text{Pa}\beta\gamma$. The increase was also detected after addition of water, implying that the unlabelled cGMP is not involved in this increase. Addition of unlabeled cGMP or water slightly diluted the mixture, ~10%; however, it is unlikely that such a small dilution could cause this increase. Modification of the $\text{Pa}\beta\gamma$ during the incubation could also be ignored because the $\text{Pa}\beta\gamma$ was pure (Fig. 3A) and the incubation was carried out on ice. Taken together, these observations deny the possibility that the increase is attributed to a reaction occurred during the incubation.

During the pre-incubation, [^3H]cGMP bound to $\text{Pa}\beta\gamma$. As another important change during the pre-incubation, the buffer in the $\text{Pa}\beta\gamma$ preparation, a phosphate buffer containing Mg^{2+} , was changed to a Tris buffer containing IBMX but not Mg^{2+} . $\text{Pa}\beta\gamma$ appears to have a tendency to change its structure in a Tris buffer, but not in a phosphate buffer (11), and Mg^{2+} binds to $\text{Pa}\beta$ (28, 29). IBMX may also increase the cGMP affinity of non-catalytic sites, as discussed later. Therefore, these changes might affect $\text{Pa}\beta\gamma$'s properties and this property change might cause to increase the level of [^3H]cGMP-bound $\text{Pa}\beta\gamma$ trapped by the filter. However, this increase was observed with either $\text{Pa}\beta\gamma$ stored in the original buffer or in the pre-incubation buffer, a Tris buffer without Mg^{2+} (data not shown). The increase was also detected with or without IBMX (data not shown). Therefore, these explanations may be disregarded. Modification of $\text{Pa}\beta\gamma$ during the pre-incubation could also be ignored, as described above. Taken together, these observations strongly suggest that [^3H]cGMP binding to $\text{Pa}\beta\gamma$ during the pre-incubation is the sole reason for the increase in the filter-trapping level of [^3H]cGMP-bound $\text{Pa}\beta\gamma$, *i.e.*, the increase appears to be caused by a conformational change on $\text{Pa}\beta\gamma$ upon binding of [^3H]cGMP.

This increase in the filter-trapping level of [^3H]cGMP-bound $\text{Pa}\beta\gamma$ was observed only after ~10-min incubation, *i.e.*, ~40-min appeared to be required to detect the increase (Fig. 5A). Why is the increase detected after such a long incubation if the increase is due to [^3H]cGMP binding? We believe that the conformational change caused by [^3H]cGMP binding progresses consistently, but slowly, and that the increase is detected only after the $\text{Pa}\beta\gamma$ having the altered conformation accumulates to a certain level. In other words, there is a threshold to trap the [^3H]cGMP-bound $\text{Pa}\beta\gamma$. We emphasize that a mechanism to accelerate

the conformational change should be present if this conformational change is indeed involved in PDE regulation.

Suppression of cGMP binding during activation of $\text{P}\alpha\beta\gamma\gamma$

Two possible stages for cGMP binding to $\text{P}\alpha\beta\gamma$ are expected in PDE regulation: during $\text{P}\alpha\beta\gamma\gamma$ activation to $\text{P}\alpha\beta\gamma$ and during $\text{P}\alpha\beta\gamma$ deactivation to $\text{P}\alpha\beta\gamma\gamma$. First we investigate whether cGMP binds to $\text{P}\alpha\beta\gamma$ during the activation of $\text{P}\alpha\beta\gamma\gamma$ to $\text{P}\alpha\beta\gamma$. After incubation of OS homogenates with GTP γ S in the presence (Fig. 2B) or absence (Fig. 2A, lower panel) of cGMP, PDE species were extracted with Buffer B and applied to a TSK-DEAE column, and their [^3H]cGMP-binding activities were measured. Both OS homogenates were incubated in the presence of IBMX and more than 20% of added cGMP remained in the cGMP-added homogenate when membranes were isolated. We found that the [^3H]cGMP binding activity of cGMP-treated $\text{P}\alpha\beta\gamma$ appeared to be slightly higher than that of cGMP-non-treated $\text{P}\alpha\beta\gamma$. However, the difference was unclear in other two studies. Therefore, we conclude that cGMP-incubated $\text{P}\alpha\beta\gamma$ has the ability to bind [^3H]cGMP similar to that in $\text{P}\alpha\beta\gamma$ obtained without cGMP. The same result was obtained when $\text{P}\alpha\beta\gamma$ was extracted with P δ in a isotonic buffer (data not shown). $\text{P}\alpha\beta\gamma$, once it binds cGMP, holds the cGMP and cannot accept [^3H]cGMP (Fig. 5B). Therefore, the [^3H]cGMP-binding activity we observed (Fig. 2B) indicates that $\text{P}\alpha\beta\gamma$ cannot bind cGMP during activation of $\text{P}\alpha\beta\gamma\gamma$ to $\text{P}\alpha\beta\gamma$.

$\text{P}\alpha\beta/\text{P}\gamma$, the minor GTP γ S-T α -activated PDE (Fig. 2), lost its [^3H]cGMP-binding activity when the fraction containing $\text{P}\alpha\beta/\text{P}\gamma$ was pre-treated with cGMP (data not shown). However, $\text{P}\alpha\beta/\text{P}\gamma$ obtained from cGMP-treated OS homogenates showed a [^3H]cGMP-binding activity (Fig. 2B) similar to that of $\text{P}\alpha\beta/\text{P}\gamma$ obtained from cGMP-non-treated homogenates (Figs. 2A, lower panel). This suggests that binding of cGMP to $\text{P}\alpha\beta/\text{P}\gamma$ is suppressed during its formation. Together, our observations indicate that the cGMP-binding activity of GTP-T α -activated PDE species is suppressed during its formation. This, we believe, is a critical finding to identify the function of cGMP binding in PDE regulation. We note that the $\text{P}\alpha\beta/\text{P}\gamma$ was eluted slightly earlier when OS homogenates were incubated with cGMP, as previously shown (20). The presence of cGMP may be crucial for the early elution; however, the real reason is unknown.

Binding of cGMP during deactivation of $\text{P}\alpha\beta\gamma$

Next, we investigated whether cGMP binds to $\text{P}\alpha\beta\gamma$ during deactivation of $\text{P}\alpha\beta\gamma$ to $\text{P}\alpha\beta\gamma\gamma$. Binding of cGMP may be involved in $\text{P}\alpha\beta\gamma$ deactivation in two ways: after interaction with $\text{P}\gamma$ and before interaction with $\text{P}\gamma$. First, we study whether cGMP binds to $\text{P}\alpha\beta\gamma$ after $\text{P}\gamma$ binding to $\text{P}\alpha\beta\gamma$. We assayed [^3H]cGMP-binding activity of $\text{P}\alpha\beta\gamma$ after incubation of $\text{P}\alpha\beta\gamma$ with $\text{P}\gamma$ or its mutants (Fig. 6). Here these $\text{P}\alpha\beta\gamma$ complexes are termed $\text{P}\alpha\beta\gamma\cdot\text{P}\gamma$ or $\text{P}\alpha\beta\gamma\cdot\text{P}\gamma$ -mutant to emphasize that [^3H]cGMP-binding activity is assayed after formation of these complexes. $\text{P}\gamma\cdot\text{GDP-T}\alpha$, instead of $\text{P}\gamma$, should be used, because $\text{P}\gamma\cdot\text{GDP-T}\alpha$, but not free $\text{P}\gamma$, is the inhibitor of $\text{P}\alpha\beta\gamma$ (13, 20). However, it is unknown whether $\text{P}\gamma$ mutants we used form a complex with GDP-T α . Therefore, free $\text{P}\gamma$ was used in this study.

1. [^3H]cGMP binding to $\text{P}\alpha\beta\gamma$ (control). The level of [^3H]cGMP binding to $\text{P}\alpha\beta\gamma$ reached a plateau in less than 2 min and was not changed during the incubation period of at least 40 min (Figs. 4B and 6B). After reaching to the plateau, ~100% of $\text{P}\alpha\beta\gamma$ bound [^3H]cGMP in the mixture. However, the plateau indicates the level of [^3H]cGMP-bound $\text{P}\alpha\beta\gamma$ trapped by the filter. In this case, the filter trapped ~16 % of the $\text{P}\alpha\beta\gamma$ existing in the mixture.
2. [^3H]cGMP binding to $\text{P}\alpha\beta\gamma\cdot\text{P}\gamma$. The level of bound [^3H]cGMP was reduced when $\text{P}\alpha\beta\gamma\cdot\text{P}\gamma$ was formed (Fig. 6A). A reason for the reduction is that binding of [^3H]cGMP to $\text{P}\alpha\beta\gamma\cdot\text{P}\gamma$ was slow and, even after 30 min incubation, did not reach

the level that $\text{Pa}\beta\gamma$ could reach in 2 min (Fig. 6B). The K_d for cGMP in $\text{Pa}\beta\gamma/\text{Py}$ is $\sim 0.33 \mu\text{M}$ (Fig. 6C), indicating that the binding of Py to $\text{Pa}\beta\gamma$ reduces its affinity for cGMP ~ 6.5 times. This reduction may be a reason for the slow binding of $[^3\text{H}]\text{cGMP}$ to $\text{Pa}\beta\gamma\cdot\text{Py}$. The efficiency of Millipore filter for trapping of $[^3\text{H}]\text{cGMP}$ -bound $\text{Pa}\beta\gamma$ was increased when Py binds to $[^3\text{H}]\text{cGMP}$ -bound $\text{Pa}\beta\gamma$, as shown below (Fig. 7A). Therefore, the reduction in the level of $[^3\text{H}]\text{cGMP}$ -binding to $\text{Pa}\beta\gamma\cdot\text{Py}$ is not due to the reduction of the Millipore filter's ability to trap the $[^3\text{H}]\text{cGMP}$ -bound $\text{Pa}\beta\gamma\cdot\text{Py}$.

3. $[^3\text{H}]\text{cGMP}$ binding to $\text{Pa}\beta\gamma\text{-C18del}$ and $\text{Pa}\beta\gamma\text{-C10del}$. Both C18sub and C10del drastically reduced the level of $[^3\text{H}]\text{cGMP}$ bound (Fig. 6A and B). A simple explanation for the reduction is that Py mutants lacking the C-terminus interrupt the entry of $[^3\text{H}]\text{cGMP}$ to a non-catalytic site in $\text{Pa}\beta\gamma$. This explanation appears to be correct because Py 's domains other than the C-terminus, especially its N-terminus, are located in the vicinity of GAF domains (30-32). However, the reduction was much larger than that caused by Py (Fig. 6A), suggesting that the way for binding of these Py mutants to $\text{Pa}\beta\gamma$ is slightly different from that of Py and that the inhibitory effect is neutralized, although partially, by its C-terminus. Alternatively, all of the $\text{Pa}\beta\gamma\text{-C-terminal-mutant}$ complexes would bind $[^3\text{H}]\text{cGMP}$; however, only the filter might trap a negligible amount of these complexes. However, the level trapped by the filter was increased when C10del was added to $[^3\text{H}]\text{cGMP}$ -bound $\text{Pa}\beta\gamma$ (Fig. 7A). Therefore, this scenario is unlikely.
4. $[^3\text{H}]\text{cGMP}$ binding to $\text{Pa}\beta\gamma\text{-N18del}$ and $\text{Pa}\beta\gamma\text{-N22del}$. The apparent level of $[^3\text{H}]\text{cGMP}$ binding was increased when $\text{Pa}\beta\gamma$ formed a complex with N18del and N22del (Figs. 6A, 6B and 6C). There are two possible explanations for this observation; 1) the level of $[^3\text{H}]\text{cGMP}$ binding to these $\text{Pa}\beta\gamma\text{-N-terminal-mutant}$ complexes was really increased, and 2) the level trapped by the filter of $[^3\text{H}]\text{cGMP}$ -bound $\text{Pa}\beta\gamma$ was increased. We compared $[^3\text{H}]\text{cGMP}$ binding to $\text{Pa}\beta\gamma$ with that to $\text{Pa}\beta\gamma\text{-N22del}$ (and $\text{Pa}\beta\gamma\text{-N18del}$). We found that 1) both $\text{Pa}\beta\gamma$ (Figs. 4A and 6C) and $\text{Pa}\beta\gamma\text{-N22del}$ (Fig. 6C) had one kind of $[^3\text{H}]\text{cGMP}$ -binding site; 2) the rate of $[^3\text{H}]\text{cGMP}$ binding to $\text{Pa}\beta\gamma\text{-N22del}$ appeared to be similar or slightly faster than that to $\text{Pa}\beta\gamma$ (Fig. 6B); and 3) the affinity of $\text{Pa}\beta\gamma\text{-N22del}$ for $[^3\text{H}]\text{cGMP}$ was higher than that of $\text{Pa}\beta\gamma$ (Fig. 6C). These findings indicate that $\text{Pa}\beta\gamma\text{-N22del}$ binds $[^3\text{H}]\text{cGMP}$ more effectively than $\text{Pa}\beta\gamma$ does. As described, $\sim 100\%$ of $\text{Pa}\beta\gamma$ rapidly binds $[^3\text{H}]\text{cGMP}$ under these conditions. Therefore, we conclude that $\sim 100\%$ of $\text{Pa}\beta\gamma\text{-N22del}$ also rapidly binds $[^3\text{H}]\text{cGMP}$ and that the apparent increase in the level of $[^3\text{H}]\text{cGMP}$ -bound $\text{Pa}\beta\gamma\text{-N22del}$ is due to the effective trapping by the filter of $[^3\text{H}]\text{cGMP}$ -bound $\text{Pa}\beta\gamma\text{-N22del}$. In other words, explanation 2, but not 1, is appropriate. The small difference in levels of $[^3\text{H}]\text{cGMP}$ binding to $\text{Pa}\beta\gamma\text{-N22del}$ and $\text{Pa}\beta\gamma\text{-N18del}$ (Fig. 7A) may also be caused by the difference in levels trapped by the filter of $[^3\text{H}]\text{cGMP}$ -bound $\text{Pa}\beta\gamma\text{-N22del}$ and $\text{Pa}\beta\gamma\text{-N18del}$.

In conclusion, Py appears to suppress $[^3\text{H}]\text{cGMP}$ binding to $\text{Pa}\beta\gamma$ by interrupting the entry of $[^3\text{H}]\text{cGMP}$ to a non-catalytic site in $\text{Pa}\beta\gamma$. This indicates that the scheme in which Py binds to $\text{Pa}\beta\gamma$ first and then cGMP binds to the $\text{Pa}\beta\gamma\gamma$ is unlikely. This also implies that Py in $\text{Pa}\beta\gamma\gamma$ interferes with cGMP release. This may be a reason for the observations that cGMPs are tightly bound to $\text{Pa}\beta\gamma\gamma$ (Fig. 3 and Ref. 12) and that release of cGMP from $\text{Pa}\beta\gamma\gamma$ is coupled with Py dissociation (Fig. 2).

Effect of Py on cGMP-bound $\text{Pa}\beta\gamma$

Next we study how Py affects $[^3\text{H}]\text{cGMP}$ -bound $\text{Pa}\beta\gamma$, *i.e.*, $\text{Pa}\beta\gamma$ having two cGMPs. Since $\sim 100\%$ of $\text{Pa}\beta\gamma$ binds $[^3\text{H}]\text{cGMP}$ and the $\text{Pa}\beta\gamma$ firmly folds $[^3\text{H}]\text{cGMP}$, we investigate the effect of Py on the filter-trapping level of $[^3\text{H}]\text{cGMP}$ -bound $\text{Pa}\beta\gamma$ (Fig. 7A). The study is

based on the result shown in Figure 5: The increase in the filter-trapping level of [^3H]cGMP-bound $\text{Pa}\beta\gamma$ is an indicator for $\text{Pa}\beta\gamma$'s conformational change by cGMP binding. $\text{Py}\cdot\text{GDP-T}\alpha$, instead of Py , should be used in this study because $\text{Py}\cdot\text{GDP-T}\alpha$, but not free Py , is the inhibitor of $\text{Pa}\beta\gamma$ (13, 20). However, as described above, it is unknown whether Py mutants we used form a complex with $\text{GDP-T}\alpha$. Therefore, free Py was used in this study.

When Py was added to [^3H]cGMP-bound $\text{Pa}\beta\gamma$ exactly in the same way as in the study shown in Figure 5A, the level trapped by the filter of [^3H]cGMP-bound $\text{Pa}\beta\gamma$ was increased in less than 15 seconds, but the trapping level of [^3H]cGMP-bound $\text{Pa}\beta\gamma$ was not changed (Fig. 7A). A simple explanation for these phenomena is that Py shortens the time required to detect the slow increase, *i.e.*, Py accelerates the conformational change of [^3H]cGMP-bound $\text{Pa}\beta\gamma$.

To strengthen the above-mentioned conclusion, the relative compactness, the Stokes radius, of cGMP-pretreated $\text{Pa}\beta\gamma$, $\text{Pa}\beta\gamma$ having two cGMPs, was also compared with or without Py (Fig. 7B). Purified $\text{Pa}\beta\gamma\gamma$ was used as $\text{Pa}\beta\gamma\gamma$ having two cGMPs because the $\text{Pa}\beta\gamma\gamma$ has two cGMPs (Fig. 3). As expected, the cGMP-pretreated $\text{Pa}\beta\gamma$ eluted as two peaks, the major peak of the $\text{Pa}\beta\gamma$ was eluted in fraction 34, and the fraction showed no [^3H]cGMP-binding activity. The important point is that $\text{Pa}\beta\gamma\gamma$ was also eluted in fraction 34 (Fig. 7B). This strongly suggests that Py does not change the relative compactness of $\text{Pa}\beta\gamma$ having two cGMPs, *i.e.*, binding of Py does not change the conformation of $\text{Pa}\beta\gamma$ having two cGMPs. Together with the observation that cGMP-binding to $\text{Pa}\beta\gamma$ slowly changes its conformation (Fig. 5) and that Py shortens the time required to detect the slow increase (Fig. 7A), the result strongly suggests that binding of Py accelerates cGMP-dependent conformational change on $\text{Pa}\beta\gamma$.

The rapid increase by Py in the filter-trapping level of [^3H]cGMP-bound $\text{Pa}\beta\gamma$ (Fig. 7A) might be due to a change by Py in its surface and/or total charge of $\text{Pa}\beta\gamma$. However, in such cases, the slow increase (Fig. 5A) could also be detected. However, the slow increase was indeed lacking. Therefore, this possibility is unlikely. We also note that the result (Fig. 7B) does not eliminate the possibility that Py binding causes a small and/or localized conformational change on the [^3H]cGMP-bound $\text{Pa}\beta\gamma$. However, the clear change in its Stokes radius (Figs. 5B) suggests that this kind of conformational change is not involved.

When C10del was added, the level trapped by the filter of [^3H]cGMP-bound $\text{Pa}\beta\gamma$ was also rapidly increased and the increase by a longer incubation (Fig. 5A) disappeared (Fig. 7A). Similar observations were also observed when N22del was added (Fig. 7A). These results suggest that binding of C10del or N22del, similar to Py binding, accelerates the conformational change on [^3H]cGMP-bound $\text{Pa}\beta\gamma$ and that a Py domain(s) located between N22 and C10 is involved in this acceleration. We note that the level increased by N22del of [^3H]cGMP-bound $\text{Pa}\beta\gamma$ was smaller than that increased by Py or C10del; however, the level increased by N18del was similar to that by Py or C10del (Fig. 7A). The level of [^3H]cGMP binding to $\text{Pa}\beta\gamma\cdot\text{N18del}$ was also consistently higher than that to $\text{Pa}\beta\gamma\cdot\text{N22del}$ (Fig. 6A). These results suggest that all or some of 4 amino-acid residues between 19-22 in Py 's amino-acid-sequence may be crucial for the acceleration.

In conclusion, these results strongly suggest that Py binding accelerates the cGMP-binding-initiated conformational change on $\text{Pa}\beta\gamma$. Together with the result showing that Py inhibits cGMP-binding to $\text{Pa}\beta\gamma$ (Fig. 6), these results also imply that the scheme in which cGMP binds to $\text{Pa}\beta\gamma$ first and then Py binds to the $\text{Pa}\beta\gamma$ is appropriate for $\text{Pa}\beta\gamma$ deactivation, the process to shift the $\text{Pa}\beta\gamma$ having one cGMP to the $\text{Pa}\beta\gamma\gamma$ having two cGMPs (Fig. 8). These results also imply a large conformational change during $\text{Pa}\beta\gamma\gamma$ activation, the process to

shift the $\text{Pa}\beta\gamma\gamma$ having two cGMPs to the $\text{Pa}\beta\gamma$ having one cGMP, although its direction is opposite to that for $\text{Pa}\beta\gamma$ deactivation (Fig. 8).

Discussion

Using bovine PDE preparations, we have recently proposed a new and comprehensive model for PDE regulation (13, 20). In this study, we try to integrate the role of non-catalytic and cGMP-specific binding sites in $\text{Pa}\beta$ in this new model. We show that $\text{Pa}\beta\gamma\gamma$, the inactive form, and $\text{Pa}\beta\gamma$, the GTP- $\text{T}\alpha$ -activated form, contain two and one cGMP, respectively, and that only $\text{Pa}\beta\gamma$ shows [^3H]cGMP binding. We also show that $\text{Pa}\beta\gamma$'s ability to bind cGMP is suppressed during formation of $\text{Pa}\beta\gamma$. We also strongly suggest that the cGMP binding slowly changes the conformation of $\text{Pa}\beta\gamma$ and that $\text{P}\gamma$ binding accelerates this conformational change. These findings are consistent with the view that $\text{Pa}\beta\gamma$ rapidly changes its conformation during its deactivation and that binding of cGMP and $\text{P}\gamma$ plays crucial roles in the conformational change (Fig. 8). These findings also imply that $\text{Pa}\beta\gamma\gamma$ rapidly changes its conformation during its activation and that the release of $\text{P}\gamma$ and cGMP plays important roles for the conformational change (Fig. 8). This is, to our understanding, the first model that the role of non-catalytic binding sites is smoothly integrated in PDE regulation.

$\text{Pa}\beta\gamma$ is the PDE species expressing [^3H]cGMP-binding activity

Identification of a PDE species expressing [^3H]cGMP-binding activity is the first step in the exploration of the role of cGMP-binding to the non-catalytic site in PDE regulation. We have shown that the GTP- $\text{T}\alpha$ -activated PDE in membranes has a high [^3H]cGMP-binding activity (Fig. 1), that $\text{Pa}\beta\gamma$ contains one cGMP, *i.e.*, $\text{Pa}\beta\gamma$ has one empty non-catalytic site, (Fig. 3), and that cGMP binds to $\text{Pa}\beta\gamma$ (Figs. 2, 3 and 4) with $K_d \sim 50$ nM (Figs. 4A and 6C). Three points related to these issues need discussion. 1) In the currently dominant model, $\text{Pa}\beta\gamma\gamma$ complexed with GTP- $\text{T}\alpha$ is believed to be the GTP- $\text{T}\alpha$ -activated PDE. Some groups also suggest that $\text{P}\gamma$ -free $\text{Pa}\beta$ is a GTP- $\text{T}\alpha$ -activated PDE. However, neither the GTP γ S- $\text{T}\alpha$ - $\text{Pa}\beta\gamma\gamma$ complex nor the $\text{P}\gamma$ -free $\text{Pa}\beta$ was detected in any OS homogenates (13, 20). Therefore, in their absence, we could not characterize the [^3H]cGMP-binding activities of these $\text{Pa}\beta$ complexes. We believe that this failure may not be crucial in exploring the role of cGMP binding in PDE regulation. 2) When [^3H]cGMP binding to $\text{Pa}\beta\gamma$ was assayed, cGMP present originally in $\text{Pa}\beta\gamma$ could be exchanged with [^3H]cGMP. However, based on the following two reasons, we conclude that the cGMP/[^3H]cGMP exchange does not occur and that the [^3H]-radioactivity we detected is only attributed to the [^3H]cGMP bound to the empty site in $\text{Pa}\beta\gamma$: 1) $\text{Pa}\beta\gamma$ had been exposed to the cGMP-free conditions more than 5 days before the final step for purification; however, the purified $\text{Pa}\beta\gamma$ contains exactly one cGMP (Fig. 3). This result suggests that $\text{Pa}\beta\gamma$ firmly holds the cGMP, *i.e.*, the cGMP is hardly exchanged with [^3H]cGMP. This is true especially when the concentration of [^3H]cGMP is as low as 1 μM . 2) Fractions containing $\text{Pa}\beta\gamma$ did not show any [^3H]cGMP-binding activity when the $\text{Pa}\beta\gamma$ was pre-treated with unlabeled cGMP (Figs. 5B and 7B). This observation indicates that neither the originally bound cGMP nor the newly bound cGMP on $\text{Pa}\beta\gamma$ can be exchanged with [^3H]cGMP in the assay mixture. We emphasize that the exchange did not occur even with 1 mM cGMP (Figs. 5A and 7A). 3) We added IBMX to suppress hydrolysis of [^3H]cGMP when [^3H]cGMP binding to $\text{Pa}\beta\gamma$ was assayed. We found that the affinity for cGMP of a PDE preparation was increased when 1 mM IBMX was present in the assay mixture (data not shown). Under these conditions, a large part of the [^3H]cGMP was not yet hydrolyzed even in the absence of IBMX, suggesting that IBMX itself affects the affinity of the PDE preparation for cGMP. IBMX also enhances the cGMP affinity of frog PDE (23). Therefore, in OS, the $\text{Pa}\beta\gamma$'s affinity for cGMP may be lower than that we observed. We note that the assay mixture for [^3H]cGMP binding did not contain

Mg²⁺ to suppress hydrolysis of [³H]cGMP. We found that both the amount of [³H]cGMP bound to Paβγ and the affinity of Paβγ for [³H]cGMP were not notably changed in the presence or absence of 5 mM MgCl₂ (data not shown). Thus, we believe that the lack of Mg²⁺ in the assay mixture does not affect the affinity of Paβγ for cGMP.

Binding of cGMP to Paβγ is not involved PDE activation

Binding of [³H]cGMP to Paβγ implies that the cGMP binding may be involved in the activation of Paβγγ to Paβγ and/or in the deactivation of Paβγ to Paβγγ. Here we show that the ability of Paβγ to bind cGMP is suppressed during the formation of Paβγγ by GTPγS-Ta in OS homogenates (Fig. 2). This indicates that the cGMP binding is not involved in the activation of Paβγγ to Paβγ. It is unknown now how this property of Paβγ is suppressed in OS homogenates containing GTPγS-Ta. However, we emphasize that either membrane-bound Paβγ (Fig. 1A) or Paβγ purified from membranes (Fig. 4A) expresses a [³H]cGMP-binding activity. This suggests that a soluble factor in the OS homogenates is involved in the suppression of cGMP binding. This factor may be GTPγS-Ta, because GTPγS-Ta, by interacting with Paβ (33, 34), may inhibit the entry of cGMP to the non-catalytic site in Paβγ. Alternatively, the interaction with GTPγS-Ta may compel Paβγ to change its conformation and the Paβγ having the altered conformation may not be able to bind cGMP. We have shown that GTPγS-Ta releases not only one Pγ but also one cGMP from Paβγγ when Paβγγ is activated (Fig. 3). The GTPγS-Ta releases Pγ by forming a complex with Pγ (13, 20); however, the mechanism to release cGMP is unknown because neither GTPγS-Ta nor Pγ can bind [³H]cGMP (Fig. 2 and Ref. 23). It is possible that this alternative mechanism may also be involved in the release of cGMP.

Binding of cGMP to Paβγ may be a mechanism for light adaptation

Our results strongly suggest that binding of cGMP, *i.e.*, formation of Paβγ having two cGMPs from Paβγ having one cGMP, changes the conformation of Paβγ. The important points are that this Paβγ's conformational change is slow (Fig. 5A); however, binding of Pγ to Paβγ having two cGMPs accelerates its conformational change (Fig. 7). Overall, these findings indicate that cGMP binds to Paβγ first and then Pγ binds to the Paβγ in Paβγ deactivation (Fig. 8). In this scheme, the residual [cGMP] is crucial for the rate of Paβγ deactivation. The residual [cGMP] in OS is dependent upon the level of illumination: If illumination is low, the residual [cGMP] is higher than the Paβγ's K_d and the cGMP-binding-dependent conformational change may be initiated on Paβγ immediately after disappearance of GTP-Ta. Thus, Paβγ will be rapidly deactivated. If illumination is high, the residual [cGMP] is lower than the K_d and the conformational change may be delayed. Therefore, Paβγ will be slowly deactivated. This may be a mechanism for light adaptation. The important point for this argument is whether the residual [cGMP] in OS can be increased to the K_d before deactivation of Paβγ. The K_d of Paβγ for cGMP we measured is ~50 nM (Figs. 4A and 6C), and the K_d in OS may be higher than ~50 nM, as described above. However, the K_d in OS should be much lower than the dark level of cGMP, 4-5 μM (2, 3). Therefore, a partial recovery of the [cGMP] may be enough to initiate the Paβγ deactivation. The [cGMP] in OS is recovered by retinal guanylate cyclase. We have recently shown that retinal guanylate cyclase is activated by a light-initiated, ATP-stimulated and Ca²⁺-sensitive mechanism (35-37) and that this activity is much higher than that expected by the current model. Indeed, it has been reported that the *in vivo* activation of retinal guanylate cyclase is much higher than that shown based on the current model (38-40). Therefore, the [cGMP] in OS may be partially recovered before complete shut down of Paβγ and the [cGMP], after partially recovered, may be enough to initiate the cGMP-binding-dependent conformational change on Paβγ.

Using N18del and N22del, we suggest that all or some of 4 amino-acid residues between 19-22 in the $P\gamma$ amino-acid-sequence may be involved in the $P\gamma$ -dependent acceleration of $P\alpha\beta\gamma$'s conformational change (Fig. 7A). This sequence includes a part of the Pro20-Xaa21-Thr22-Pro23-Arg24 sequence, the sequence essential for phosphorylation of Thr22 by cyclin-dependent protein kinase 5 (41-45). It is unknown now whether Thr22 phosphorylation affects the $P\gamma$ -dependent acceleration of $P\alpha\beta\gamma$'s conformational change; however, this study is very interesting. Comparison with PDE5 (25-27) will also be of great interest for future studies.

Previous models for the role of cGMP binding in PDE regulation

It should be emphasized that all previous studies, including our studies, did not identify PDE species expressing cGMP-binding activity. For frog PDE, PDE species exiting in OS membranes (14-16, 21, 22, 33) or PDE species treated with trypsin (15) were used. For bovine PDE, PDE species treated with trypsin were used (17, 18). Therefore, the cGMP-binding activity reported might be the activity ascribed by the mixture of PDE species and/or affected by other PDE species. Moreover, the cGMP-binding activity obtained might be affected by different rates of filter trapping. Therefore, conclusions in previous studies may not be correct. For example, previous studies suggested that $P\gamma$ stimulated cGMP binding to GTP- $T\alpha$ -activated frog PDE/membranes (21, 22). We propose to reevaluate this conclusion. Trypsin-treated PDE species have other problems: Trypsin digests one PDE species in various ways, and the trypsin-treated PDE appears to lose not only all $P\gamma$ s but also all cGMPs (17, 18). Presence of such PDE species in OS is doubtful (13, 20).

Two previous models to explain the role of non-catalytic site in PDE regulation, the cGMP-regulated $P\alpha\beta$ - $P\gamma$ interaction model (14-18) and the cGMP-binding-direct regulation model (19), were also based on unclear identification of the PDE species expressing cGMP-binding activity. For example, the former model implies the presence of GTP- $T\alpha$ - $P\alpha\beta\gamma\gamma$ complex containing two cGMPs or the presence of GTP- $T\alpha$ - $P\alpha\beta\gamma\gamma$ complex containing one or no cGMP and cGMP-binding to the complex. However, neither the presence of these complexes nor cGMP binding to the complex have been verified. In addition, the mechanism by which GTP- $T\alpha$ activates $P\alpha\beta\gamma\gamma$ without changing the interaction between $P\alpha\beta$ and $P\gamma$ has not been shown. The former model also implies that GTP- $T\alpha$ releases $P\gamma$ when the [cGMP] in OS is low or absent and that the released $P\gamma$ accelerates deactivation of GTP- $T\alpha$ -activated PDE. These implications are also problematical; 1) $P\gamma$ is released from $P\alpha\beta\gamma\gamma$ to accelerate PDE deactivation. This appears to be a self-contradiction; 2) cGMP is released from $P\alpha\beta\gamma\gamma$ when [cGMP] is low or absent. However, as shown previously (12) and now, this is not the case in bovine PDE; 3) $P\gamma$ is released from GDP- $T\alpha$. However, $P\gamma$ forms a tight complex with GDP- $T\alpha$ (33, 46, 47), and the mechanism to release $P\gamma$ from the complex is unknown {We have shown that $P\gamma$ -GDP- $T\alpha$, without $P\gamma$ liberation, inhibits $P\alpha\beta\gamma$ (13)}; and 4) they indicate, to our understanding, that the released $P\gamma$ enhances GTPase activity of the $T\alpha$ that is already complexed with $P\gamma$ in $P\alpha\beta\gamma\gamma$. However, this mechanism is unknown. In the latter model, there is no data showing which PDE species binds cGMP, how the cGMP-binding changes the PDE catalytic activity and how GTP- $T\alpha$ is involved in the mechanism. Therefore, it is difficult to integrate these models in PDE regulation. We and another group (48-50) have also proposed a model in which the non-catalytic sites serve as a cytoplasmic buffer for cGMP. This model appears not to be directly related to PDE regulation, and thus we do not discuss here.

Experimental procedures

Materials

Dark-adapted frozen bovine retinas were obtained from J. A. Lawson Co. (Lincoln NE). [^3H]cGMP was from PerkinElmer Life Sciences. Other chemical reagents and materials were purchased from various sources described in previous articles (13, 20).

Preparation of OS membranes and isolation of PDE species

Membranes were prepared from OS homogenates as described (13). PDE species were solubilized with a hypotonic buffer or P δ in an isotonic buffer and purified by using DEAE and molecular sieve columns (13, 20). In each study, protein-contents in OS homogenates and volumes of buffers used to wash membranes or to extract PDE species were slightly different. These differences have been shown not to be critical (13, 20). 1) *Use of a hypotonic buffer*. Details have been published (20). Here, as an example, the procedure for the experiment shown in Figure 2 is described: OS homogenates (~50 mg protein) were suspended in 20 ml Buffer A (20 mM HEPES, pH 7.5, 5 mM DTT, 5 mM MgCl₂, 5 μM leupeptin, 5 μM pepstatin A, 0.1 mM PMSF, 1 mM benzamidine, and 150 mM NaCl) and divided into three portions. To each portion, cGMP (final 1 mM), GTP γ S (final 50 μM), or cGMP + GTP γ S was added. After overnight incubation on ice, membranes were isolated and washed ($\times 2$) with 7 ml Buffer A supplemented with cGMP (1 mM), GTP γ S (50 μM), or cGMP + GTP γ S. Finally, all membranes were washed with 7 ml Buffer A ($\times 2$). PDE species were extracted from these membranes with 7 ml Buffer B (5 mM Tris-HCl, pH 7.5, 5 mM DTT, 0.5 mM MgCl₂, 5 μM leupeptin, 5 μM pepstatin A, 0.1 mM PMSF and 1 mM benzamidine) ($\times 7$) and applied to a TSK-DEAE 5PW column. After assaying PDE activity and identifying a 88-kDa-protein-doublet by SDS-PAGE, fractions containing PDE species were concentrated to ~0.5 ml and applied to a Superdex 200 HR column. The 88-kDa-doublet has been identified as P $\alpha\beta$ (13, 20). When cGMP was added, IBMX (final 1 mM) was also added to all portions. 2) *Use of P δ in an isotonic buffer*. Details of this procedure have been reported (20). After incubation of OS homogenates with or without GTP γ S, membranes were isolated and washed as described above. These membranes were suspended in Buffer C (10 mM Na-phosphate, pH 6.8, 5 mM DTT, 5 mM MgCl₂, 5 μM leupeptin, 5 μM pepstatin A, 0.1 mM PMSF, 1 mM benzamidine, 100 mM NaCl), incubated with P δ (final 3 μM) overnight on ice, and washed with Buffer C ($\times 2$) (13, 20). All supernatants were combined together and applied to a TSK-DEAE 5PW column. P $\alpha\beta\gamma\gamma$ and P $\alpha\beta\gamma$ are isolated as P $\alpha\beta\gamma\gamma\delta$ and P $\alpha\beta\gamma\delta\delta$, respectively (20). We also obtained a PDE preparation using a Bio-Gel A-0.5 m column (9 $\times$ 600 mm) that had been equilibrated with Buffer D (10 mM Na-phosphate, pH 6.8, 1 mM DTT, 2 mM MgCl₂, 5 μM leupeptin, 5 μM pepstatin A, 0.1 mM PMSF, 1 mM benzamidine, 150 mM NaCl and 15% glycerol). Proteins were eluted with Buffer D (Fig. 1B and C). The chromatography conditions were: flow rate, 0.8 ml/13 min; and fraction volume, 0.7 ml. The resin was chosen because the resin does not have any charge and only a small amount of proteins may stick to the resin.

Preparation of P γ and its mutants

Bovine P γ and its mutants, N18del (a P γ mutant in which 18 amino-acids in the N-terminus were deleted), N22del (a P γ mutant in which 22 amino-acids in the N-terminus were deleted), C18Sub (a P γ mutant in which 18 amino-acids in the C-terminus were substituted with a frame-shift mutation), and C10del (a P γ mutant in which 10 amino-acids in the C-terminus were deleted), were used. Oligonucleotides (49) and procedures to express and purify P γ and these P γ mutants (51) have been reported.

Contents of cGMP in $\text{Pa}\beta\gamma\gamma$ and $\text{Pa}\beta\gamma$

Contents of cGMP bound to purified $\text{Pa}\beta\gamma\gamma$ and $\text{Pa}\beta\gamma$ were measured using a cGMP immunoassay kit, the Correlate-EIA Direct Cyclic GMP Enzyme Immunoassay Kit (Assay Designs, Ann Arbor, MI). After incubation of 100 μl of these preparations with 900 μl 0.1 M HCl for 2 h on ice, supernatants were obtained by centrifugation ($350,000 \times g$, 20 min, 4°C) and diluted with 0.1 M HCl. The amount of cGMP in these mixtures (100 μl) was measured as the instruction of the kit indicated. Apparent M_r s of $\text{Pa}\beta\gamma\gamma$ (216,894) and $\text{Pa}\beta\gamma$ (207,226) were used for the calculation of cGMP concentrations.

Determination of [^3H]cGMP-binding activity in PDE

Two methods were used. 1) *Filter assay*. This assay was performed as described (23, 30). Typically, $\text{Pa}\beta\gamma$ ($\sim 15 \mu\text{g}$ before purification, and $\sim 2 \mu\text{g}$, after purification) was incubated with 0.5 or 1 μM [^3H]cGMP (1 mCi/ml) in the assay medium (final volume, 100 μl) containing 25 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 1 mM IBMX for 30 min on ice, and 80 μl aliquots were applied to a Millipore filter (HA, pore size 0.45 μm) that had been wetted with 20 mM sodium phosphate buffer (pH 6.5). The filter was washed with 3 ml of phosphate buffer ($\times 3$) and dissolved in 4 ml of scintillation cocktail. When 1 μM [^3H]cGMP was used, the maximum level ($\sim 1.7\%$ of the added counts) was reached in 2 min and the binding was stable for at least 40 min (Fig. 4B). Addition of a 1000-fold excess of unlabeled cGMP to the [^3H]cGMP inhibited [^3H]cGMP-binding by greater than 99%. A linear relationship existed between the level trapped by the filter of [^3H]cGMP-bound $\text{Pa}\beta\gamma$ and the protein level. 2) *Gel filtration*. This method was used to estimate the efficiency of the Millipore filter to trap [^3H]cGMP-bound $\text{Pa}\beta\gamma$. After incubation with 1 μM [^3H]cGMP in 0.5 ml of 25 mM Tris-HCl, pH 7.5, containing 0.1 mM EDTA and 1 mM IBMX for 30 min on ice, $\text{Pa}\beta\gamma$ (40-90 μg) was applied to a Superdex 200 HR column that had been equilibrated with Buffer D. The chromatography conditions were: room temperature, 0.3-0.5 ml/min flow rate and 0.3-0.5 ml fraction volume. The level of [^3H]cGMP bound to $\text{Pa}\beta\gamma$ was calculated based on the [^3H]-radioactivity in 50-70 μl of the fraction. The fraction (50-70 μl) was also applied to a Millipore filter and the [^3H]-radioactivity on the filter was measured. The efficiency of the filter's ability to trap [^3H]cGMP-bound $\text{Pa}\beta\gamma$ was estimated by comparison of these [^3H]-radioactivities.

Determination of Stokes radii of $\text{Pa}\beta\gamma$ treated with or without cGMP

Purified $\text{Pa}\beta\gamma$ (70 μg) was incubated with or without unlabeled cGMP (0.5 mM) in 0.5 ml of 25 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 1 mM IBMX for 30 min on ice. After concentration to $\sim 0.25 \text{ ml}$, the mixture was applied to a Superdex 200 HR column which had been equilibrated with Buffer E (10 mM Tris-HCl, pH 7.5, 1 mM DTT, 2 mM MgCl_2 , 5 μM leupeptin, 5 μM pepstatin A, 0.1 mM PMSF, 1 mM benzamidine, 100 mM NaCl and 10% glycerol). The chromatography conditions were: room temperature, 0.25 ml/min flow rate and 0.25 ml fraction volume. PDE activity was assayed using 5-20 μl of the fraction. [^3H]cGMP-binding activity was measured using 50 μl of the fraction. Neither IBMX nor cGMP was added to the elution buffer, Buffer E, because IBMX may change $\text{Pa}\beta\gamma$'s affinity for cGMP, as discussed in this study, and cGMP, once bound to $\text{Pa}\beta\gamma$, is not dissociated. The apparent Stokes radii of these PDE species were calculated as described (25-27). The following proteins were used to standardize the column: ovalbumin, 30.5 $\text{\AA}$; conalbumin, 40.4 $\text{\AA}$; aldolase, 48.1 $\text{\AA}$; and thyroglobulin, 85.0 $\text{\AA}$.

Analytical procedures

PDE activity was assayed as described (30). Protein concentration was measured with bovine serum albumin as the standard (52). SDS-PAGE using 8-16% gradient gels was carried out as described (53). Protein staining with Coomassie blue was also performed as

described (54). The advantage of this staining method has been described in previous reports (13, 20). The method was also used to estimate roughly the content of $\text{P}\alpha\beta$ in gels. In our results, individual points represent the average values of duplicate assays. All experiments were carried out at least three times and the results were similar. The data shown are representative of these experiments.

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Abbreviation

PDE	cGMP phosphodiesterase
OS	outer segments of retinal photoreceptors
Pα and Pβ	rod PDE catalytic subunits
Pγ	rod PDE inhibitory subunit
Pα'	cone PDE catalytic subunit
Pγ'	cone PDE inhibitory subunit
P$\alpha\beta$/Pγ	P $\alpha\beta$ complexes having an unknown number of P γ
Pδ	a prenyl-binding protein
M_r	molecular weight
Tα	transducin α subunit
T$\beta\gamma$	transducin β and γ subunits
GTP-Tα	GTP-bound T α
GDP-Tα	GDP-bound T α
GTPγS	guanosine 5'-O-(3-thiotriphosphate)
GTPγS-Tα	GTP γ S-bound T α
PAGE	polyacryamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
IBMX	1-methyl-3-isobutylxanthine

Figure 1.

Binding of [3 H]cGMP to membranous PDE. **A.** Levels of [3 H]cGMP binding to OS membranes treated with or without GTP γ S. OS homogenates (27.5 mg protein) were suspended in 18.4 ml of Buffer A and divided into two portions. After incubation of a portion with 50 μ M GTP γ S overnight on ice, its membranes were washed with 5 ml Buffer A supplemented with 50 μ M GTP γ S ($\times 2$) and 5 ml Buffer A ($\times 2$) and suspended in 5 ml Buffer A. The other portion was treated in the same way but without GTP γ S. Binding of [3 H]cGMP to these suspensions (10 μ l) was assayed using 1 μ M [3 H]cGMP. **B** and **C.** [3 H]cGMP binding to proteins extracted from OS membranes treated with or without GTP γ S. OS homogenates (27.7 mg protein) were suspended in 18 ml of Buffer A, divided into two portions and treated with or without GTP γ S. Proteins were extracted from membranes with 3 ml Buffer B ($\times 7$), concentrated to ~ 0.5 ml and applied to Bio-Gel A-0.5 m column. [3 H]cGMP-binding activity (**B**) and PDE activity (not shown) were assayed using 60 and 5 μ l of the fraction, respectively. Protein profiles in the fraction (90 μ l) were analyzed by SDS-PAGE and staining with Coomassie blue (**C**). The left end lane shows M_r of standard proteins, 94, 67 and 43 kDa.

Figure 2.

Binding of [³H]cGMP to PDE species extracted from OS membranes. **A** and **B**. PDE species extracted with a hypotonic buffer. Details of the procedure are in Experimental procedures. OS homogenates (50.4 mg protein) were suspended in 20 ml Buffer A and divided into three portions. After incubation with cGMP (**A**, upper panel), GTP γ S (**A**, lower panel) or cGMP + GTP γ S (**B**), proteins were extracted with Buffer B (a hypotonic buffer), applied to a TSK-DEAE 5PW column and eluted. Fractions containing PDE species were determined by SDS-PAGE and assaying PDE activity. Elution profiles of the 88-kDa-protein, P $\alpha\beta$, are shown in each panel. Elution profile of other proteins was shown in Ref 20. PDE species were identified as described (20). Binding of [³H]cGMP to the fraction (60 μ l) was measured with 0.5 μ M [³H]cGMP. **C**. PDE species extracted with P δ in an isotonic buffer. OS homogenates (12.4 mg) were suspended in 13 ml of Buffer A and divided into two portions. After incubation of a portion with GTP γ S (50 μ M) for 1 h on ice, membranes were washed with 2 ml of Buffer A containing GTP γ S (50 μ M) and 2 ml of Buffer A. The other portion was treated in the same way but without GTP γ S. These membranes were suspended in 2.5 ml of Buffer D, incubated with P δ (final 3 μ M) overnight on ice, and washed with 2 ml of Buffer D ($\times 2$). All supernatants were collected and applied to a TSK-DEAE 5PW column. Rod and cone PDE species and their stoichiometry and transducin subunits were identified as described (20). Binding of [³H]cGMP to the fraction (50 μ l) was measured with 0.5 μ M [³H]cGMP (upper panel). Protein profiles in fractions (40 μ l) were analyzed by SDS-PAGE and staining with Coomassie blue (lower panel). Due to the limited space, only results from GTP γ S-treated membranes are shown. Profiles of PDE species from GTP γ S-non-treated membranes are in Ref. 20.

Figure 3. Levels of cGMP contained in Pαβγγ and Pαβγ. Pαβγγ (6.50 μg/20 μl) and Pαβγ (4.75 μg/50 μl) were purified from GTPγS-treated OS homogenates. **A.** Purity of these PDE preparations. Preparations of Pαβγγ (10 μl) and Pαβγ (25 μl) were applied to SDS-PAGE followed by staining with Coomassie blue. **B.** Levels of cGMP contained in these PDE species. Contents of cGMP were measured using a cGMP immunoassay kit.

Figure 4.

Binding of [³H]cGMP to Pαβγ. **A.** Concentration of [³H]cGMP. [³H]cGMP binding to Pαβγ (1.92 μg) was measured with indicated concentrations of [³H]cGMP. The [³H]cGMP-binding activity was analyzed by Scatchard plotting (Insert). **B.** Time-course. Pαβγ (17.3 μg) was incubated in 55 mM Tris-HCl, (pH 7.5) containing 4.4 mM EDTA and 1.1 mM IBMX (final volume, 720 μl) on ice for 10 min. The [³H]cGMP binding was initiated by adding 80 μl of 10 μM [³H]cGMP. After incubation for indicated periods, an aliquot (80 μl) was taken and applied to a Millipore filter. **C.** The cyclic nucleotide-specificity. After incubation of Pαβγ (1.92 μg) with indicated concentration of unlabeled cGMP (●) or cAMP (○) on ice for 10 min, [³H]cGMP binding was measured with 1 μM [³H]cGMP. The 100% activity indicates that 1.46 pmol [³H]cGMP bound to Pαβγ in tubes. **D.** Levels of [³H]cGMP-bound Pαβγ trapped by the filter. OS homogenates (18.9 mg protein) were suspended in 9.7 ml of Buffer A. After isolation by the TSK-DEAE 5PW column chromatography and concentration to 0.3 ml, the Pαβγ preparation (~80 μg) was incubated with 1 μM [³H]cGMP for 30 min on ice and applied to a TSK 250 column that had been equilibrated with Buffer D. The level of [³H]cGMP bound to Pαβγ was calculated based on the [³H]-radioactivity in 70 μl of the fraction (●). The fraction (70 μl) was also applied to a Millipore filter and the [³H]-radioactivity on the filter was measured (□). Only fractions containing Pαβγ were shown. Insert: The rate of [³H]-radioactivity on the filter per the level of [³H]-radioactivity in the fraction. The 100% radioactivity indicates the [³H]-radioactivity detected in fraction 15. Fraction 15 (70 μl) contained 3.2 μg Pαβγ (15.5 pmol) and 13.1 pmol of [³H]cGMP.

Figure 5.

Change of $\text{P}\alpha\beta\gamma$'s characteristics by cGMP binding. **A.** Dissociation of $[^3\text{H}]$ cGMP bound to $\text{P}\alpha\beta\gamma$. Purified $\text{P}\alpha\beta\gamma$ (16.0 μg) suspended in 640 μl of 55.5 mM Tris-HCl (pH 7.5) containing 4.44 mM EDTA and 1.11 mM IBMX, and $[^3\text{H}]$ cGMP binding was initiated by adding 80 μl of 9 μM $[^3\text{H}]$ cGMP. After incubation for 30 min on ice, an aliquot (72 μl) was withdrawn and applied to a Millipore filter, and its radioactivity was designated as the level at time 0. Simultaneously, 72 μl of 10 mM unlabeled cGMP (●) or water (○) was added to the assay mixture. After incubation for 0.25, 0.5, 0.75, 1, 2, 5, 10, and 20 min, an aliquot (80 μl) was withdrawn and applied to a Millipore filter, and its $[^3\text{H}]$ -radioactivity was measured. The arrow indicates the addition of cGMP or water. The 100% activity indicates that 1.32 pmol of $[^3\text{H}]$ cGMP was detected in 1.6 μg of $\text{P}\alpha\beta\gamma$ (7.72 pmol). **B.** Elution profile of $\text{P}\alpha\beta\gamma$ from a gel filtration column. Purified $\text{P}\alpha\beta\gamma$ (70 μg) was incubated with (black) or without (red) unlabeled cGMP (0.5 mM) in 0.5 ml of 25 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 1 mM IBMX for 30 min on ice and applied to a Superdex 200 HR column that had been equilibrated with Buffer E. Detailed conditions for this elution are in Experimental Procedure. PDE activity was assayed using 5 μl of the fraction (●). The 100% PDE activity indicates that 12.5 nmol cGMP hydrolyzed/min/tube. $[^3\text{H}]$ cGMP binding activity was measured using 50 μl of the fraction (□). The 100% activity indicates that 1.50 pmol of $[^3\text{H}]$ cGMP was detected in the assay mixture.

Figure 6.

Effects of P γ and its mutants on [3H]cGMP binding to Pa $\beta\gamma$. **A.** The effect on the level of [3H]cGMP binding. After incubation of Pa $\beta\gamma$ (1.92 μ g) with various concentrations of P γ or its mutants, the [3H]cGMP-binding activity was measured. The 100% activity indicates that 1.46 pmol [3H]cGMP bound to Pa $\beta\gamma$ in tubes. Following P γ and its mutants were used: ●, wild type P γ ; □, N18del; △, N22del; ▲, C18Sub, and ▼, C10del. **B.** The effect on the time-course of [3H]cGMP-binding. Pa $\beta\gamma$ (17.3 μ g) was incubated with 1.11 μ M P γ or its mutants in 55 mM Tris-HCl, (pH 7.5) containing 4.4 mM EDTA and 1.1 mM IBMX (final volume, 720 μ l) on ice for 30 min. The [3H]cGMP binding was initiated by adding 80 μ l of 10 μ M [3H]cGMP. After incubation for indicated periods on ice, an aliquot (80 μ l) was taken and applied to a Millipore filter. Following P γ and its mutants were used: ○, control; ●, wild type P γ ; △, N22del; and ▲, C18Sub. **C.** The effect on the Scatchard plot. Pa $\beta\gamma$ (1.92 μ g) was incubated with 1 mM of wild type P γ (●) or N22del (△). As a control, Pa $\beta\gamma$ alone was incubated (○). Then, [3H]cGMP binding was initiated by adding indicated concentrations of [3H]cGMP (**C-1**). The [3H]cGMP binding in C-1 is analyzed by Scatchard plotting (**C-2**).

Figure 7.

Py effect on the conformational change of $P\alpha\beta\gamma$ by cGMP binding. **A.** The time to detect the increase in the level of $[^3H]cGMP$ -bound $P\alpha\beta\gamma$ trapped by the filter. The experiment was carried out as a part of the study depicted in Figure 5A. $P\alpha\beta\gamma$ (16.0 μg) was suspended in 640 μl of 55.5 mM Tris-HCl (pH 7.5) containing 4.44 mM EDTA and 1.11 mM IBMX, and $[^3H]cGMP$ -binding to the $P\alpha\beta\gamma$ was initiated by adding 80 μl of 9 μM $[^3H]cGMP$. After incubation for 30 min, an aliquot (72 μl) was withdrawn and applied to a Millipore filter, and its radioactivity was designated as the level at time 0. Simultaneously, a mixture (72 μl) of **Py** or its mutant (10 μM) with or without cGMP (10 mM) was added to the assay mixture: (●), with cGMP; and (○), without cGMP. After incubation for 0.25, 0.5, 0.75, 1, 2, 5, 10, and 20 min, an aliquot (80 μl) was withdrawn and applied to a Millipore filter, and its radioactivity was measured. The arrow (↑) indicates the addition of **Py** (or **Py** mutant) with or without cGMP. The arrow (⇌) indicates levels of $[^3H]cGMP$ -bound $P\alpha\beta\gamma$ with (▲) or without (Δ) N18del. The 100% activity indicates that 1.32 pmol of $[^3H]cGMP$ was detected in 1.6 μg of $P\alpha\beta\gamma$ (7.72 pmol). Data shown in Figure 5A was used as a control for this study. **B.** Elution profile of PDE species from a gel filtration column. Purified $P\alpha\beta\gamma$ (70 μg) was incubated with unlabeled cGMP (0.5 mM) in 0.5 ml of 25 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 1 mM IBMX for 30 min on ice and applied to a Superdex 200 HR column that had been equilibrated with Buffer E (black). The chromatography conditions are in Experimental Procedure. PDE activity (●) was assayed using 5 μl of the fraction. $[^3H]cGMP$ -binding activity (□) was measured using 50 μl of the fraction. $P\alpha\beta\gamma$ (90 μg) was also applied to the column and eluted in the same manner (red). PDE activity was assayed using 20 μl of the fraction (●). The 100% PDE activity indicates that 12.1 nmol cGMP hydrolyzed/min/tube. $[^3H]cGMP$ -binding activity was measured using 50 μl of the fraction (□). The 100% $[^3H]cGMP$ -binding activity indicates that 2.4 pmol of $[^3H]cGMP$ was detected in the assay mixture.

Figure 8.

Role of the non-catalytic cGMP-binding site in PDE regulation. *Activation.* GTP- $T\alpha$ activates $P\alpha\beta\gamma\gamma/2cGMPs$ ($P\alpha\beta\gamma\gamma$ having two cGMPs) to $P\alpha\beta\gamma/cGMP$ ($P\alpha\beta\gamma$ having one cGMP). At the initial stage, cGMP is present in OS; however, binding of cGMP to the empty site on $P\alpha\beta\gamma/cGMP$ is suppressed. *Deactivation.* After hydrolysis of cGMP, retinal guanylate cyclase initiates to produce cGMP from GTP. When the [cGMP] in OS is increased to ~50 nM, the cGMP binds to $P\alpha\beta\gamma/cGMP$ and $P\alpha\beta\gamma\gamma/2cGMPs$ is formed. Then, the PDE species slowly changes its conformation. Interaction with $P\gamma \cdot GDP-T\alpha$ accelerates the conformational change and swiftly establishes the inactive form of PDE ($P\alpha\beta\gamma\gamma/2cGMPs$).