Communication

Possible Stimulation of Retinal Rod Recovery to Dark State by cGMP Release from a cGMP Phosphodiesterase Noncatalytic Site*

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Cyclic GMP phosphodiesterase, a key enzyme for photontransduction, contains α, β (Pαβ), and two γ (Pγ) subunits. In addition to catalytic sites, Pαβ has two classes of noncatalytic cGMP binding sites with different affinities (K_a values <100 nM and >1 μM). Pγ regulates Pαβ as an inhibitor of cGMP hydrolysis and as a stimulator of cGMP binding to the high affinity noncatalytic sites. Pγ release from Pαβ in the GTP-bound subunit of transducin (GTPγα) interrupts these two functions. Here we describe a novel regulation of the Pγ release by [cGMP] and its physiological implication. We isolated Pγ mutants that exhibit abnormally one of these two functions, indicating the distinct domains in Pγ are involved to express these functions. When [cGMP] was high (~5 μM), Pγ responsible for the inhibition of cGMP hydrolysis was preferentially released, and cGMP hydrolysis activity of Pαβ was increased about 10 times. When [cGMP] was low (less than ~0.5 μM), Pγ responsible for the stimulation of cGMP binding to the high affinity sites was released. The Pγ release resulted in the decrease of relative affinity of cGMP for the high affinity sites to at least 1/10, followed by the rapid release of cGMP from one of the high affinity sites (apparent K_a ~3.8 μM). cGMP (~5 μM) inhibited the extraction of Pαβ from rod membranes by a Mg^{2+}-free hypotonic buffer. The inhibition of Pαβ extraction was not affected by Pγ, suggesting that Pαβ detects on the order of micromolar [cGMP] using low affinity noncatalytic sites on Pαβ. Because [cGMP] is ~5 μM in darkness and lowered by photoexcitation and phosphodiesterase concentration is ~30 μM in rod photoreceptors, it is possible that cGMP phosphodiesterase functions to increase cytoplasmic [cGMP] after [cGMP] is reduced to the illuminated level.

Since Bitensky and Miller suggested the involvement of cyclic nucleotides in phototransduction (1), many investigators have contributed to establish the role of cGMP in phototransduction (1–3). The illuminated rhodopsin stimulates GTP/GDP exchange on Tz, which in turn activates cGMP phosphodiesterase. The resulting decrease of cytoplasmic [cGMP] leads to closure of cGMP-gated channels and hyperpolarization of photoreceptors. The closure of channels also blocks Ca^{2+} influx, while Ca^{2+} efflux by a Na^+/Ca^{2+} exchanger continues. The resulting decline in the free [Ca^{2+}] is believed to play a major role in the adaptation and recovery processes of photoreceptors by negative feedback regulation by [Ca^{2+}] (4). However, regulation of phototransduction by the decrease in cytoplasmic [cGMP] has never been clarified.

Rod cGMP phosphodiesterase contains α, β (Pαβ), and two γ (Pγ) subunits. Previous studies have shown that Pαβ has two catalytic sites for cGMP hydrolysis (5, 6) as well as two classes of noncatalytic cGMP binding sites with different affinities (K_a values ~100 nM and 1–6 μM) (5–9). These noncatalytic sites are the major cGMP binding sites in ROS, binding more than 90% of the cellular cGMP (7, 9). The roles of these noncatalytic sites in phototransduction still remain unclear. However, it is clear in amphibian ROS that Pγ regulates Pαβ not only as an inhibitor of cGMP hydrolysis (10) but also as a stimulator of cGMP binding to the high affinity noncatalytic sites (11–13). The same, nonmodified Pγ shows these functions (13). These two Pγ functions are interrupted when GTPγα dissociates Pγ from Pαβ (10–13). By manipulating ionic strength in the reaction mixtures, we have recently suggested that Pγ expresses only one of these functions when Pγ is complexed with Pαβ (13).

In this study, we refer to the Pγ responsible for the inhibition of cGMP hydrolysis as iPγ and the Pγ responsible for the stimulation of cGMP binding to the high affinity sites as sPγ. Using data showing that release of these Pγs is regulated by [cGMP], we propose a novel mechanism for the recovery of [cGMP] to the dark level.

**EXPERIMENTAL PROCEDURES**

ROS membranes from dark-adapted Rana catesbiana, Pγ-less (activated) cGMP phosphodiesterase membranes, and frog Pγ were prepared as described (10). Activity of phosphodiesterase was measured as described (10, 13). Amounts of Pγ released by GTPγTα were determined using a Pγ-specific antibody (13). The Pγ content measured by the Pγ-specific antibody was similar to the value obtained by the inhibition of cGMP hydrolysis (10, 13). Equilibrium binding of [3H]cGMP to Pαβ in various ROS membranes was assayed as described (7, 13). To ensure [3H]cGMP binding to the high affinity noncatalytic sites on Pαβ, 0.5 μM [3H]cGMP (12) was used in the presence of 1 mM IBMX and 2 mM EDTA. Under these conditions, ~5% (at most) of added cGMP was hydrolyzed. We have already shown that recombinant bovine Pγ has the same functions as frog Pγ (13). To measure [3H]cGMP release from the high affinity sites on Pαβ, [3H]cGMP was loaded to the sites on Pαβ. Then, [3H]cGMP release was measured with or without 100 μM GTPγS using a filter

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method (11) without nonradioactive cGMP. The filter was washed with 10 mM Tris-HCl (pH 7.5) and 2 mM EDTA (×4). Each washing took about 2 s. Data were analyzed with a nonlinear curve-fitting program Igor Pro (Wave Metrics, Lake Oswego, OR) (14). All experiments were carried out more than three times, and the results were similar. Data shown were the representative of these experiments.

In order to replace C-terminal 18 amino acids or N-terminal 16 amino acids, a frameshift mutation was introduced into bovine Pγ cDNA (15). For the C-terminal frameshift mutation, the following primers were used for the first PCR reaction: up, 5′-GTCATCTGTTTGG-GAGGCCCTCA-3′ and down, 5′-CCGCGGTTCGATCTTAACTATGC-3′. To amplify the mutant Pγ gene, the second PCR reaction was carried out with the purified product from the first PCR reaction and the following primer: 5′-GGAGTTTTTACATGCTTGAACCA-3′. The mutant contains the following amino acid sequence in its C-terminal: FGRSTTWRCTRWPRTAS. For the N-terminal frameshift mutation, the following primers were used for the first PCR reaction: up, 5′-ACAAGCATATGAAACCGGGTGCCACCCAAGGC-3′ and down, 5′-GAGGAATGACGGGCCCCCCATCACC-3′. To amplify the mutant Pγ gene, the second PCR reaction was carried out with the purified product from the first PCR reaction and the following primer: 5′-GGGGTCG-GATCCTAGATGCGCTACTG-3′. The mutant contains the following amino acid sequence in its N-terminal: MKPGATQGRDPVGHKGD. These amplified Pγ mutant gene products were digested with NdeI and BamHI and cloned into a NdeI-BamHI-digested pET-IIA (Novagen). The vector was transferred to Escherichia coli BL21 (DE3) (Novagen) for expression of Pγ. The mutations were confirmed by double-stranded DNA sequencing using the fmol DNA sequencing system (Promega). The protein expression was induced by addition of 1 mM isopropyl DNA sequencing using the fmol DNA sequencing system (Promega).

RESULTS AND DISCUSSION

Pγ Mutants Devoid of One of Two Functions—Pγ regulates Pαβ as iPγ and sPγ. We isolated Pγ mutants in which one of these functions was suppressed (Fig. 1). When the C-terminal 18 amino acids were replaced by a frameshift mutation, the mutant (C-18) stimulated cGMP binding similarly to wild type Pγ; however, the inhibitory activity was completely abolished. When the N-terminal 16 amino acids were replaced, the mutant (N-16) retained inhibitory activity like wild type Pγ. However, a 200–300 mM amount of this mutant Pγ was required to reach 50% of the maximal cGMP binding that was achieved with ∼10 mM wild type Pγ. These observations indicate that different domains in Pγ are involved to express these functions. Because Pγ expresses only one of these functions when Pγ is complexed with Pαβ (Ref. 13 and as shown below), these data suggest that Pγ binds to different sites on Pαβ to express these functions. It should be emphasized that a C-terminal-deleted (10 amino acids) mutant lost the iPγ activity as shown by many groups; however, the mutant also showed less stimulatory activity for the cGMP binding due to less interaction with Pαβ (data not shown). We also note that stimulation of cGMP binding by the C-18 mutant clearly indicates that high phosphodiesterase activity did not affect cGMP binding to the high affinity noncatalytic sites on Pαβ by reducing [cGMP], since cGMP hydrolysis was maximal for all concentrations of the mutant, while cGMP binding was dependent upon the mutant concentration.

Low Sensitivity of the iPγ Release to cGMP—Phosphodiesterase is activated by GTP-Tα through iPγ release from Pαβ in amphibian ROS membranes (10, 16, 17). We have shown that the iPγ release can be distinguished from sPγ release by changing [NaCl] in a releasing buffer (0–200 mM) (13). However, the change in the cytoplasmic ionic strength by light in frog ROS is not so drastic (18). We have investigated the possibility that cGMP is a physiological regulator for the release of functionally different Pγ from Pαβ. cGMP (1 mM) inhibited the total Pγ release regardless of [NaCl] in the releasing buffer (Fig. 2A). When the buffer contained less than 100 mM NaCl, the phosphodiesterase activity in membranes washed without cGMP was consistently higher than that washed with cGMP (Fig. 2B). When the buffer contained 150 mM NaCl, the maximum activation was achieved by the first washing without cGMP; however, in the presence of cGMP the full activation was accomplished after the third washing (Fig. 2C). These data unexpectedly indicate that the release of iPγ, i.e. the phosphodiesterase activation, is sensitive to cGMP. However, we emphasize that, in the presence of 150 mM NaCl, 60% of the maximum phosphodiesterase activity can be retained by the first washing even in the presence of 1 mM cGMP (Fig. 2C). Because less than 5 μM cGMP is hydrolyzed for photoexcitation (2), the suppression of phosphodiesterase activation by cGMP may be negligible under the physiological ionic conditions (18). We note that the release of Tα was not affected by cGMP and that excess amounts of Tα were released for the Pγ release in each washing (data not shown). We also found that ∼5 μM cGMP inhibited the total Pγ release in a buffer containing 150 mM NaCl and 1 mM IBMX (data not shown). This result is consistent with data published by Arshavsky et al. (17). However, we used 1 μM cGMP in this study to exclude the effect of cGMP hydrolysis by phosphodiesterase activated variously.

In the absence of cGMP, the amount of Pγ released with 200 mM NaCl was 3.4 ± 0.5 times (n = 3) that of Pγ released without NaCl (Fig. 2A). Measurement of Pγ by the inhibition of cGMP hydrolysis also indicated that the amount of Pγ released with 200 mM NaCl was 3.6 ± 0.9 times (n = 3) that of Pγ released without NaCl (data not shown). However, membranes washed with or without NaCl showed the activated, same level of phosphodiesterase activity (Fig. 2B). These observations indicate that only 30% (at most) of the total Pγ released functions as iPγ, and more than 70% of Pγ released is not responsible for phosphodiesterase inhibition.
These membranes were washed with 0.5 ml of Buffer A (10 mM Tris-Cl, 5 mM DTT, 5 mM MgCl₂, 0.1 mM CaCl₂ and 0.1 mM PMSF) (B). Py was extracted by adding Buffer A alone (C). These membranes were washed further with 1.5 ml of Buffer A (×3) and suspended in 1 ml of Buffer A. Equilibrium ³HcGMP binding to these membranes (6.7 µl) was measured in the presence of various amounts of frog Py, B. cGMP release. Illuminated ROS membranes (15 mg) were washed with 2 ml of Buffer A (×7) and suspended in 1.5 ml of a buffer (10 mM Tris-Cl, 1 mM DTT, 2 mM EDTA, and 0.1 mM PMSF). These membranes (560 µg) were incubated with 1.0 µM ³HcGMP in 280 µl of a buffer (40 mM Tris-Cl (pH 7.5), 4 mM EDTA, and 2 mM IBMX) for 30 min at 20 °C. Then, 280 µl of a solution (300 mM NaCl and 10 mM MgCl₂) with (●) or without (○) 200 µM GTP·S was rapidly added and mixed. After incubation for indicated periods at 20 °C, an aliquot (80 µl) was applied to a Millipore filter (HA, pore size 0.45 µm) on a vacuum manifold system, and the filter was washed. Filter-bound radioactivity was measured and analyzed. After 5-min incubation, more than 95% of ³HcGMP added was still present in the reaction mixture containing GTP·S.

At present, we do not have a simple explanation for such an unequivocal release of the functionally different Py. We speculate that a portion of Py bound Paβ, especially if Py, may be GTP-Tα-insensitive (10) due to its phosphorylation (19). Phosphorylated Py cannot be released by GTP-Tα due to the loss of affinity for GTP-Tα (20).

High Sensitivity of the sPy Release to cGMP—When the releasing buffer contained more than 100 mM NaCl, the ratio of the total Py released in the absence of cGMP to the Py released in the presence of cGMP was approximately 2 to 1 (Fig. 2A). However, phosphodiesterase activities in these membranes measured after Py release were identical (Fig. 2B). These observations indicate that 50% (at least) of the total Py released is sensitive to cGMP and that the Py is not iP. When ROS membranes were washed with a buffer containing only GTP, equilibrium ³HcGMP binding to the high affinity noncatalytic sites on Paβ in these membranes was increased by Py added (Fig. 3A). However, if the membranes were washed with a buffer containing GTP and cGMP, approximately 80% of the maximum ³HcGMP binding was destroyed without adding Py and the added Py barely stimulated the ³HcGMP binding. Both membranes had the same level of phosphodiesterase activity (data not shown). Five µM cGMP (with 1 mM IBMX) produced a similar effect, although the effect was smaller due to the possible hydrolysis of cGMP during washing (data not shown). These observations indicate that, when [cGMP] is high, iP, but not sPy, is released readily. When [cGMP] is low, both iP and sPy are released.

Without Py, [³H]cGMP could not be loaded to the high affinity sites on Paβ using 1 µM [³H]cGMP. However, in the presence of Py, various concentrations of [³H]cGMP (0.1, 0.5, and 1.0 µM) showed the similar level of equilibrium cGMP binding to the sites (data not shown). We note that cGMP binding with a K₅ value ~1 µM can be measured under our conditions (7). These data indicate that the relative affinity of cGMP for the high affinity sites is reduced to 1/10 (at least) by release of sPy. We measured the time course of cGMP release from the high affinity sites on Paβ by GTP-Tα (Fig. 3B). Without GTP·S, [³H]cGMP, which had been loaded to the sites, was not released; however, with GTP·S, biphasic dissociation kinetics were observed (apparent t₁/₂ = 3.8 and 181 s, 20 °C). These results suggest that two different high affinity sites are present on Paβ and that GTP·S·Tα rapidly dissociates [³H]cGMP from one of the sites. We note that ~0.5 µM [³H]cGMP was present during the [³H]cGMP release, and nonradioactive cGMP was not added to chase [³H]cGMP from these sites. Thus, the real dissociation rate of [³H]cGMP may be faster than that measured under our conditions. Cote et al. (12) have reported the
that under their conditions the release of sP
indicated, in the presence of 1 mM IBMX (17.5 mg) were washed with 2 ml of Buffer A (×7) and then these membranes were divided into eight portions, and P
were divided into two portions. Each portion was washed with 1.5 ml of Buffer A containing 400 mM NaCl (0.5 ml of Buffer A (×7) and then these membranes were divided into two portions. Each portion was washed with 2 ml of Buffer A containing 400 mM NaCl in the presence (C) or absence (B) of 1 mM cGMP. Then, the portion was further divided into three portions. P
was extracted from each portion by 0.5 ml of Buffer B with various concentrations of cGMP, as indicated, in the presence of 1 mM IBMX (×3). P
in each supernatant (0.3 ml) was isolated by SDS-gel electrophoresis.

**Possible Role of the Low Affinity Noncatalytic Sites on P
— We studied the possibility that P
detects [cGMP] by its low affinity noncatalytic cGMP binding site. When P
was extracted from ROS membranes with a magnesium-free hypotonic buffer, cGMP specifically inhibited the P
release (Fig. 4A). cAMP (100 μM) did not inhibit the release (data not shown). This inhibition was detectable as 5 μM cGMP and was not dependent upon P
, since this inhibition was not changed even after membranes were washed with GTP or GTP (Fig. 4B and C). These data indicate that P
detects cGMP binding sites by photoaffinity labeling (6, 10,

the K
value for cGMP binding to low affinity noncatalytic sites is about 1–6 μM (6, 8), and the K
value of the Pβ catalytic sites is about 0.3–1 μM. On the basis of these data, we propose that the low affinity noncatalytic sites on Pβ serve as a sensor of the cytoplasmic [cGMP]. The binding of cGMP to these low affinity sites appears to inhibit the sP
release by GTP-To from Pβ through changing of P
conformation, because a similar [cGMP] inhibits the release of sP
as described above.

The present data indicate that sP
is released by GTP-To when [cGMP] becomes low, resulting in the rapid release of cGMP from one of the high affinity noncatalytic sites on Pβ. We hypothesize that this cGMP release functions to promote recovery of [cGMP] to the dark level in amphibian ROS. We emphasize that hydrolysis of GTP bound to Tα measured by biochemical methods is slow (21) and that GTP-To is still present after the turnoff of GTP-To-activated phosphodiesterase by P
phosphorylation (19, 20). Therefore, GTP-To is expected to be present at the late stage of phototransduction. GTP-To may not release iP
at the stage. We found that release of iP
by GTP-To is inhibited by endogenous ADP-riboseylation of Pβ complexed with Pβ (22). We speculate that this kind of mechanism may be functional when [cGMP] becomes low. Since [cGMP] is ~5 μM in darkness and Pβ is estimated to be ~30 μM in concentration, cGMP released from one of the high affinity sites by GTP-To may be enough to increase cytoplasmic [cGMP] to the dark level, and less than 1.2 s is required to release ~5 μM cGMP from the site. Thus, it is possible that cGMP phosphodiesterase functions to increase cytoplasmic [cGMP] to the dark level when [cGMP] is reduced to the illuminated level. At present, we cannot compare the contribution of cGMP release from the high affinity site on Pβ to that of cGMP synthesis by guanylyl cyclase for the recovery of [cGMP] to the dark level, because the precise data of the velocity of cGMP synthesis are not available.

**References**
1. Miller, W. H. (1990) Invest. Ophthalmol. & Visual Sci. 31, 1664–1673
2. Pugh, E. N., Jr., and Lamb, T. D. (1987) Invest. Ophthalmol. & Visual Sci. 28, 34–49
3. Pugh, E. N., Jr., and Lamb, T. D. (1993) Biochem. Biophys. Acta 1141, 111–149
4. Keutels, Y., and Yau, K.-W. (1996) Trends Neurosci. 19, 73–81
5. Ovchinnikov, Y. A., Gubanov, V. V., Khramtsov, N. V., Iachenko, K. A., Zagranichny, V. E., Moradov, K. G., Shuraeva, T. M., and Lipkin, V. M. (1987) FEDS Lett. 223, 169–173
6. Lipkin, V. M., Khramtsov, N. V., Vasilevskaya, I. A., Atabekova, N. V., Moradov, K. G., Gubanov, V. V., Li, T., Li, T., Johnston, J. P., Volpp, K. J., and Applebury, M. L. (1990) J. Biol. Chem. 265, 12955–12959
7. Yamazaki, A., Sen, I., Bitesnys, M. W., Camellie, J. E., and Greengard, P. (1980) J. Biol. Chem. 255, 11619–11624
8. Gillespie, P. G., and Beavo, J. A. (1988) J. Biol. Chem. 263, 8133–8141
9. Cote, R. H., and Brunnock, M. A. (1985) J. Biol. Chem. 260, 17190–17198
10. Yamazaki, A., Hayashi, F., Tatsumi, M., Bitensky, M. W., and George, J. S. (1983) J. Biol. Chem. 258, 8119–8124
11. Yamazaki, A., Bartz, F. A., Chernoff, N., and Bitensky, M. W. (1982) J. Biol. Chem. 257, 7302–7306
12. Cote, R. H., Bownds, M. D., and Arshavsky, V. Y. (1994) Proc. Natl. Acad. Sci. U. S. A. 70, 3702–3706
13. Yamazaki, A., Bartz, F. A., Chernoff, N., and Bitensky, M. W. (1982) J. Biol. Chem. 257, 7302–7306
14. McPherson, R. H., Bownds, M. D., and Arshavsky, V. Y. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4945–4949
15. Yamazaki, A., Yamazaki, M., Bondarenko, V. A., and Matsumoto, H. (1996) Biochem. Biophys. Res. Commun. 222, 483–493
16. McPherson, G. A. (1985) J. Pharmcol. Methods 14, 213–228
17. Ovchinnikov, Y. A., Lipkin, V. M., Kumarev, V. P., Gubanov, V. V., Khramtsov, N. V., Akhmedov, N. B., Zagranichny, V. E., and Muradov, K. G. (1986) Biochem. Biophys. Res. Commun. 141, 598–603
18. Tsuboi, S., Matsumoto, H., Jackson, K. W., Tsujimoto, K., Williams, T., and Yamazaki, A. (1990) J. Biol. Chem. 265, 24501–24507
19. Sembly, A. P., and Wala, B. (1985) J. Physiol. (Lond.) 358, 183–195
20. Tsuboi, S., Matsumoto, H., and Yamazaki, A. (1994) J. Biol. Chem. 269, 15016–15023
21. Ting, T. D., and Ho, Y.-K. (1991) Biochemistry 30, 8996–9007
22. Bitensky, M. W., and George, J. S. (1993) Invest. Ophthalmol. & Visual Sci. 37, 328