THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 35, Issue of August 29, pp. 33150–33160, 2003
Printed in U.S.A.

A Critical Role for ATP in the Stimulation of Retinal Guanylyl Cyclase by Guanylyl Cyclase-activating Proteins*

Received for publication, April 9, 2003, and in revised form, May 27, 2003
Published, JBC Papers in Press, June 10, 2003, DOI 10.1074/jbc.M303678200

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It has been believed that retinal guanylyl cyclase (retGC), a key enzyme in the cGMP recovery to the dark state, is solely activated by guanylyl cyclase-activating proteins (GCAPs) in a Ca^{2+}-sensitive manner. However, a question has arisen as to whether the observed GCAP stimulation of retGC is sufficient to account for the cGMP recovery because the stimulated activity measured *in vitro* is less than the light/GTP-activated cGMP phosphodiesterase activity. Here we report that the retGC activation by GCAPs is larger than previously reported and that a preincubation with adenine nucleotide is essential for the large activation. Under certain conditions, ATP is two times more effective than adenylyl imidodiphosphate (AMP-PNP), a hydrolysis-resistant ATP analog; however, this study mainly used AMP-PNP to focus on the role of adenine nucleotide binding to retGC. When photoreceptor outer segment homogenates are preincubated with AMP-PNP (EC_{50} = 0.65 ± 0.20 mM), GCAP2 enhanced the retGC activity 10–13 times over the control rate. Without AMP-PNP, GCAP2 stimulated the control activity only 3–4-fold as in previous reports. The large activation is due to a GCAP2-dependent increase in V_{max} without an alteration of retGC affinity for GCAP2 (EC_{50} = 47.9 ± 2.7 nM). GCAP1 stimulated retGC activity in a similar fashion but with lower affinity (EC_{50} = 308 nM). In the AMP-PNP preincubation, low Ca^{2+} concentrations are not required, and retGC exists as a monomeric form. This large activation is accomplished through enhanced action of GCAPs as shown by Ca^{2+} inhibition of the activity (IC_{50} = 178 nM). We propose that retGC is activated by a two-step mechanism: a conformational change by ATP binding to its kinase homology domain under high Ca^{2+} concentrations that allows large enhancement of GCAP activation under low Ca^{2+} concentrations.

Photoexcitation of rhodopsin results in hydrolysis of cGMP by PDE[1] in retinal photoreceptors. The decrease in cytoplasmic cGMP concentrations leads to closure of cGMP-gated channels, blockade of Na^+ influx, and hyperpolarization of photoreceptor plasma membranes (1–3). The reduction of cGMP-gated channel activity also blocks Ca^{2+} influx and allows Na^+/Ca^{2+}, K^+ exchangers to decrease cytoplasmic Ca^{2+} concentrations from ~500 nM to near 30 nM in photoreceptor OS (4–6). The decrease in free Ca^{2+} concentrations acts as a trigger for cGMP synthesis (7, 8). Two membrane guanylyl cyclases, retGC-1 and -2 (also referred to as ROS GC-1 and -2 or GC-E and GC-F, respectively), are involved in the cGMP synthesis in photoreceptor OS (9–14). It has been shown that retGC-1 is localized in the photoreceptor layer and that cone OS contains more retGC-1 than rod OS (15, 16). In photoreceptor OS, retGC-1 appears to be associated with the marginal region of disk membranes and/or the plasma membranes (15). retGC-2 is localized in photoreceptors (12); however, detailed localization of retGC-2 in retina has not been reported.

RetGC shares an overall molecular configuration similar to those of peptide-regulated GCs and contains an extracellular domain, a transmembrane domain, a kinase homology domain (KHD), and a catalytic domain (17, 18). However, it has been believed that regulation of retGC is different from those of peptide-regulated GCs. For example, GC-A, a typical peptide-regulated GC, is activated through binding of atrial natriuretic peptide to the extracellular domain (17, 18). In retGC, the extracellular domain appears not to be required for the stimulation, although its real function is unknown. Instead, the Ca^{2+}-sensitive stimulation of retGC is mediated by binding of calmodulin-like Ca^{2+}-binding proteins termed GCAPs (19, 20) to the intracellular domains (21, 22). Three GCAPs have been reported (19, 20, 23) and two GCAPs, 1 and 2, have been extensively studied. The mechanism of retGC activation by GCAPs is not clear; however, dimerization (or oligomerization) of retGC appears to be involved (24–27). It is also known that retGC is activated by S100 proteins, other Ca^{2+}-binding proteins, at high Ca^{2+} concentrations (28, 29). This regulation is believed to be involved in retinal cells other than photoreceptor OS. It should be noted that the relationship between GCAP localization and their functions appears to be controversial. Immunocytochemical analysis (30, 31), with genetic analysis of cone degeneration (32, 33), suggests that GCAP1 is primarily expressed in cones, whereas GCAP2 is primarily detected in rods and, at a lower level, in cones. Very recent studies using double knockout mice ($GCAP^{-/-}$) showed, as expected, that overexpression of bovine GCAP2 in $GCAP^{-/-}$ rescued the Ca^{2+} sensitivity of retGC and the time for the rod recovery from saturating flashes (34, 35). However, the GCAP2 overexpression could not restore the normal kinetics of response evoked by

* This work was supported in part by National Institutes of Health Grant EY09631 and an unrestricted grant from Research to Prevent Blindness. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PDE, cGMP phosphodiesterase; retGC, retinal guanylyl cyclase; GC, membrane-bound guanylyl cyclase; KHD, kinase homology domain in GCs; OS, outer segments of retinal photoreceptors; ROS, rod outer segments; GCAPs, retGC-activating proteins; AMP-PNP, adenylyl imidodiphosphate; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; HPLC, high pressure liquid chromatography.

subsaturating flashes (34). On the contrary, Howes *et al.* (36) reported that GCAP1 expression in GCAP$^{-/-}$ restored the wild type properties of rod light response in the absence of GCAP2. They proposed that GCAP1 supports the generation of wild type flash responses in rods. In these studies using double knockout mice, it is not clear whether the expression of bovine GCAPs in mouse rods completely restores the function of the missing mouse GCAPs and whether the overexpression of GCAP2 disturbs normal functions. Moreover, it is not known whether all GCAPs expressed in GCAP$^{-/-}$ function properly and whether GCAP$^{-/-}$ can restore all normal properties if both GCAPs are expressed.

There is another crucial difference between peptide-regulated GCs and retGC. ATP is obligatory in the stimulation of GC-A (17, 18, 37). However, ATP has not been reported to be essential for the GCAP stimulation of retGC. ATP has been believed to only modify retGC activity; low ATP concentrations (less than ~0.5 mM) slightly stimulate and high ATP concentrations (more than ~1 mM) significantly inhibit retGC activity in OS membranes (38–41). Several studies also reported the synergetic effect of ATP and GCAPs on retGC activity (21, 41–43), although the ATP concentrations used in these studies (less than 0.5 mM) were lower than the physiological ATP concentrations in rods (3–4 mM) (44), and the activation was not large in OS membranes. It should be emphasized that the significance of the inhibition of retGC by physiological ATP concentrations has been completely ignored in the previous studies of retGC regulation.

There is another fundamental question ignored in the previous retGC studies: whether the observed GCAP-stimulated retGC activity is sufficient to account for the recovery of cGMP level to the dark state. Needless to say, the activity of purified retGC is less than that of light/GTP-stimulated PDE (45–48). Sitaramayya *et al.* (39) estimated, based on published data obtained *in vitro* and their measurements of retGC activities *in vitro*, that the maximal rate of cGMP hydrolysis in light/GTP-activated PDE is 7–200 times greater than the potential retGC activity. In addition, the content of retGC in rods is not larger than that of PDE, although these estimations varied (from 1:1 to 1:10) (46–48). In addition, recent studies on GCAPs$^{-/-}$ mice showed that the mean single photon response amplitude was nearly five times larger than that of wild type rods (34, 35, 49). At all flash strengths examined, the light-evoked PDE activity measured as the rate of rise of the signal at early times was the same in GCAP$^{-/-}$ and control rods. These observations suggest that the level of GCAP-stimulated retGC activity *in vivo* may be similar or higher than that of light/GTP-activated PDE. Other electrophysiological studies also estimated the retGC activity *in vivo* as being higher than that measured *in vitro* (50, 51). These studies imply that there may be another mechanism for the retGC activation and/or that unknown components may be involved in the further stimulation of retGC by GCAPs *in vivo*.

In this study, we have attempted to reconcile the difference in retGC activities observed *in vivo* and *in vitro*. We show that pretreatment of OS homogenates with adenine nucleotides (1–10 mM) enhances the level of retGC activity stimulated by GCAPs. Based on these observations, we propose a new mechanism for retGC activation.

EXPERIMENTAL PROCEDURES

Materials—Frozen dark-adapted retinas were purchased from J. A. Lawson Co. (Lincoln, NE). Okadaic acid and AMP-PNP were purchased from Sigma. TSKgel DEAE-2SW column (4.6 mm × 25 cm) was obtained from TosoHaas. [^{125}I]Anti-rabbit IgG whole antibody from goat was obtained from PerkinElmer Life Sciences. Sources of other materials were described previously (25). GCAP1 and -2 were kind gifts from Drs. Rameshwar K. Sharma (University of Medicine and Dentistry of New Jersey) and Alexander M. Dizhoor (Pennsylvania College of Optometry), respectively. Antibodies against retGC-1 and GCAP2 were kindly provided from Drs. Fumio Hayashi (Kobe University, Kobe, Japan) and Alexander M. Dizhoor, respectively.

Preparation of retGC Samples—OS preparations used in this study were isolated from bovine retinas without separation of rod OS from cone OS. Thus, the OS preparations were mixtures of rod and cone OS, although ROS is expected to present mainly in the preparations. In addition, retGC-1 was not separated from retGC-2 in the preparations. Therefore, the retGC activity described here is the activity measured as the total activity of retGC-1 and –2, although the retGC-1 activity is expected to be dominant due to the low contents of retGC-2 (12). Bovine OS were prepared from dark-adapted retinas as described (52). GCAP-free membranes were prepared as described previously (25) with minor modifications. Briefly, OS isolated from 25 retinas were suspended in 5 ml of Buffer A (5 mM HEPES, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 100 μM $CaCl_2$, 0.1 mM PMSF, 5 μM leupeptin, and 5 μM pepstatin A), homogenized by passing a needle with a 21-gauge diameter (seven times) and centrifuged (100,000 × *g*, 15 min, 4 °C). The process was repeated seven times. The membrane fraction was further washed in 5 ml of Buffer B (5 mM HEPES, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 2 mM EGTA, 0.1 mM PMSF, 5 μM leupeptin, and 5 μM pepstatin A). The process was repeated seven times. The membrane fraction (5 mg/ml) was suspended in Buffer C (10 mM HEPES, pH 7.5, 1 mM DTT, 2 mM $MgCl_2$, 0.1 mM PMSF, 5 μM leupeptin, and 5 μM pepstatin A) and stored at −70 °C.

Preincubation of retGC with Adenine Nucleotides—An OS preparation (150 μg) was homogenized in 300 μl of Buffer D (20 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 0.1 mM PMSF, 5 μM leupeptin, and 5 μM pepstatin A) and incubated with 5 mM AMP-PNP. The preincubation was carried out on ice for 30 min unless otherwise noted. After centrifugation (100,000 × *g*, 10 min, 4 °C), the membrane fraction was washed in 750 μl of buffer E (10 mM HEPES, pH 7.5, 1 mM DTT, 100 μM $CaCl_2$, 0.1 mM PMSF, 5 μM leupeptin, and 5 μM pepstatin A) containing 5 mM AMP-PNP (x3) and then 750 μl of Buffer F (10 mM HEPES (pH 7.5), 1 mM DTT, 5 mM $MgCl_2$, 2 mM EGTA, 0.1 mM PMSF, 5 μM leupeptin, and 5 μM pepstatin A) to exclude AMP-PNP (two times). The membrane fraction was suspended in 270 μl of Buffer F and used as retGC. Preincubation of OS homogenates with ATP (see Fig. 1) was carried out under slightly different conditions. Details of these conditions are described in the figure legend. We estimate that the residual concentration of AMP-PNP in the membrane fraction was less than 20 μM. This estimate is based on experiments using the radioactive tracers [^{8}H]cGMP and [^{125}I]anti-rabbit IgG whole antibody from goat. This estimation was also confirmed by HPLC using TSKgel DEAE-2SW column as described below. We also found that the AMP-PNP used was contaminated with ~5% ADP. However, preincubation of OS homogenates with 5 mM ADP only increased retGC activity ~15% as compared with OS membranes incubated without ADP, suggesting that the ADP contamination in AMP-PNP was not involved in the activation.

Identification of Nucleotides—To estimate nucleotide concentrations in samples, the nucleotides were separated by HPLC, and their amounts were determined using the peak areas. The samples were prepared by centrifugation (100,000 × *g*, 30 min, 4 °C). After appropriate dilution, a portion of the supernatant was applied to a TSKgel DEAE-2SW column that had been equilibrated with Solution A (CH_3CN in 0.1 M phosphate, pH 3.0, 20/80). The column was washed with 5 ml of Solution A, and the nucleotides were eluted by a 30-min linear gradient from Solution A to B (CH_3CN in 0.5 M phosphate, pH 3.0, 20/80). The column chromatography was carried out with the flow rate 1.0 ml/min, and the nucleotides were detected by using a UV detector at 260 nm. The following are retention times of nucleotides measured under our conditions: cGMP, 7.6 min; ADP, 13.2 min; AMP-PNP, 18.8 min; GDP, 20.2 min; ATP, 21.6 min; and GTP, 28.9 min.

Construction of Three-dimensional Models of KHD of retGC-1—The amino acid sequence of retGC-1 (bovine guanylyl cyclase D; SwissProtein code P55203) was aligned using Clustal X (53) with the catalytic domains of four protein kinases for which crystal structures have been determined. The KHD sequence of retGC-1 (residues 536–830 using the Swiss Protein sequence numbers) as predicted by the multiple sequence alignment was then modeled using the Swiss PdbViewer 3.7b2 in conjunction with the Swiss Model program (54). For modeling of the unstimulated conformation of retGC1, the coordinates of the nonactivated insulin receptor kinase domain (Protein Data Bank code 1IRK) were used as the template; fitting of the model to the template gave a root mean square deviation of 0.40 Å for 1080 carbon-α atoms. For the AMP-PNP-stimulated conformation, the coordinates for the activated insulin receptor kinase domain with bound AMP-PNP (Pro-

tein Data Bank code 1IR3) were used; fitting gave a root mean square value of 0.56 Å for 1030 carbon-α atoms.

Measurement of retGC Activity—The activity of retGC was measured as described (25, 46, 55). Under the assay conditions, the hydrolysis of cGMP formed was negligible, and a linear relationship exists between the retGC activity measured and the protein amounts used. The linear relationship was established even in the highly activated retGC (see Fig. 2*B*). All of the results about retGC activities were analyzed using the computer program Prism (GraphPad). We note that the concentration of protein used for the enzyme assay is expressed as total protein rather than the rhodopsin concentration frequently used for retGC activity. We also note that the protein concentration measured before the adenine nucleotide preincubation was used to calculate the enzyme activities. This calculation underestimates the actual specific activity of membrane-bound retGC because it includes soluble proteins that are subsequently removed by membrane washes. Measurements of protein concentrations indicated that ~82% of the total proteins in OS homogenates were membrane-bound, implying that the actual enzyme activity may be ~20% higher than the activity shown.

Other Analytical Methods—Dimerization of retGC was monitored using a cross-linker, bis(sulfosuccinimidyl) suberate, and Western blotting with a retGC-1-specific antibody (25). SDS-PAGE was performed as described (56). Protein concentration was measured with bovine serum albumin as standard (57). Ca^{2+}-EGTA buffers were prepared as described (25). It should be emphasized that individual points obtained in all experiments represent the average values of duplicate assays and that all experiments were carried out more than two times, and the results were similar. The data shown are representative of these experiments.

RESULTS

Pretreatment of OS Homogenates with Adenine Nucleotides Enhances Both Control and GCAP2-stimulated retGC Activities—When OS homogenates were preincubated with 1 mM ATP (37 °C, 30 min) and soluble components were washed out, GCAP2-stimulated retGC activities in membrane fractions were high (Fig. 1). This ATP activation was ~10 times larger than the GCAP-stimulated retGC activities in GCAP-free membranes (47.6–56.2 as compared with 5.44 nmol cGMP/min/mg formed in the presence of 500 nM GCAP2). Such large activation of retGC was totally unexpected. We also found that the preincubation in the presence of high Ca^{2+} concentrations was more effective than in its low concentration (56.2–47.6 nmol cGMP/min/mg formed in the presence of 500 nM GCAP2). It should be emphasized that the activity is expressed as specific activity rather than the activation level compared with that obtained without ATP and that GCAP2-stimulated retGC activities in GCAP-free membranes were measured without the preincubation. Thus, the large activation by ATP is not due to the reduction of retGC control activity by instability of retGC/membranes incubated without ATP (58). Indeed, the GCAP2 sensitivity of retGC in OS homogenates preincubated without ATP was less than that in GCAP-free membranes, suggesting the instability of retGC/membranes incubated without ATP. These observations strongly suggest that ATP is positively involved in the stimulation of retGC by GCAP2.

However, mechanisms underlying the ATP stimulation are difficult to elucidate because under these conditions ATP may affect retGC activity in various ways. For example, ATP may express its functions by binding to retGC. ATP may also be a phosphate donor in the phosphorylation of proteins involved in the retGC regulation. We note that phosphatase inhibitors were added to the ATP preincubation. We also found that OS washed membranes contained high ATP hydrolytic activity under retGC assay conditions (33 °C, 10 min, 5–10 μg of protein/200 μl). For example, ~60% of 5 mM ATP was hydrolyzed to ADP under our assay conditions, even with an ATP-regenerating system present. This high ATP hydrolytic activity makes data interpretation difficult. For example, 0.1–0.5 mM ATP was added to the assay mixture in some previous studies; however, the final ATP concentrations may be substantially lower. In addition, it is possible that ATP metabolites may affect retGC activity.

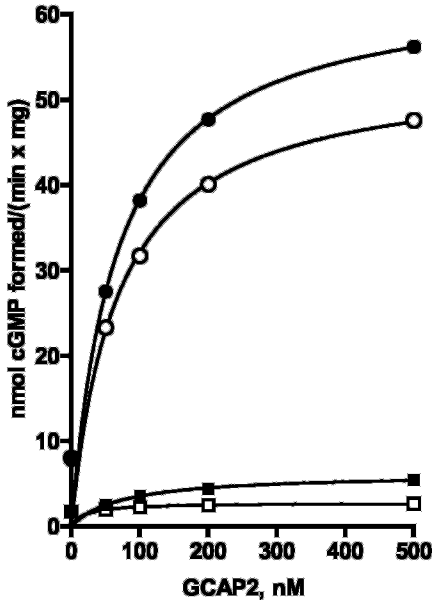

FIG. 1. **GCAP2-stimulated retGC activity after preincubation of OS homogenates with ATP.** OS homogenates (100 μg) were preincubated 200 μl of buffer D containing 10 nM okadaic acid and 10 mM NaF with (● and ○) or without (□) 1 mM ATP (37 °C, 30 min). The preincubation was also carried out in the presence of high (●) or low (○ and □) Ca^{2+} concentrations. For the high Ca^{2+} concentrations, 100 μM $CaCl_2$ was added; for the low concentrations, 1 mM EGTA was added. The membrane fraction was washed with 500 μl of the same buffer (without ATP) using centrifugation (100,000 × *g*, 20 min, 4 °C) (three times) and resuspended in the same buffer (1 mg/ml). RetGC activity (5 μg/100 μl) was measured with indicated concentrations of GCAP2 in the presence of 1 mM EGTA. The effect of GCAP2 on retGC activity in GCAP-free membranes is also shown (■). GCAP-free membranes were not preincubated. The enzyme activities shown are the averages of two experiments.

To focus on the ATP binding to retGC, as a first step in elucidating the ATP stimulation, and to exclude other possibilities and difficulty in data interpretation, we preincubated the OS homogenate (on ice, 30 min) with AMP-PNP (Fig. 2). AMP-PNP, a hydrolysis-resistant ATP analog, has been considered to bind to retGC but not to serve as a phosphate donor in protein phosphorylation (58). The use of ice temperature prevented the inactivation of retGC previously shown in incubation at 30–37 °C (58). We found that retGC activity, measured without GCAPs, was increased slightly but consistently by the AMP-PNP preincubation (from 1.56–2.10 to 2.99–3.15 nmol of cGMP/min/mg formed). Without AMP-PNP, GCAP2 at 500 nM stimulated three to four times the control activity of retGC (from 1.56–2.10 to 6.28–6.94 nmol cGMP/min/mg formed). However, with 5 mM AMP-PNP, 500 nM GCAP2 stimulated the control activity about 10–13 times (from 1.56–2.10 to 20.6–21.4 nmol cGMP/min/mg formed). In the GCAP2-stimulated activity, a linear relationship existed between retGC activity and membranes added (Fig. 2*B*). These results show that the AMP-PNP preincubation also enhances GCAP-stimulated retGC activity, although the activation level was less than that by the ATP preincubation (Fig. 1). These observations strongly suggest that binding of adenine nucleotide may be involved in the large activation of retGC by GCAP2. It should be emphasized that the preincubation of OS homogenates with GTP slightly enhanced the GCAP2-stimulated retGC activity; however, the level of the enhancement was ~10% of those obtained by the AMP-PNP preincubation (data not shown). This indicates that adenine nucleotides are required for this activation. We also note that GCAP2 (500 nM) activated retGC in the

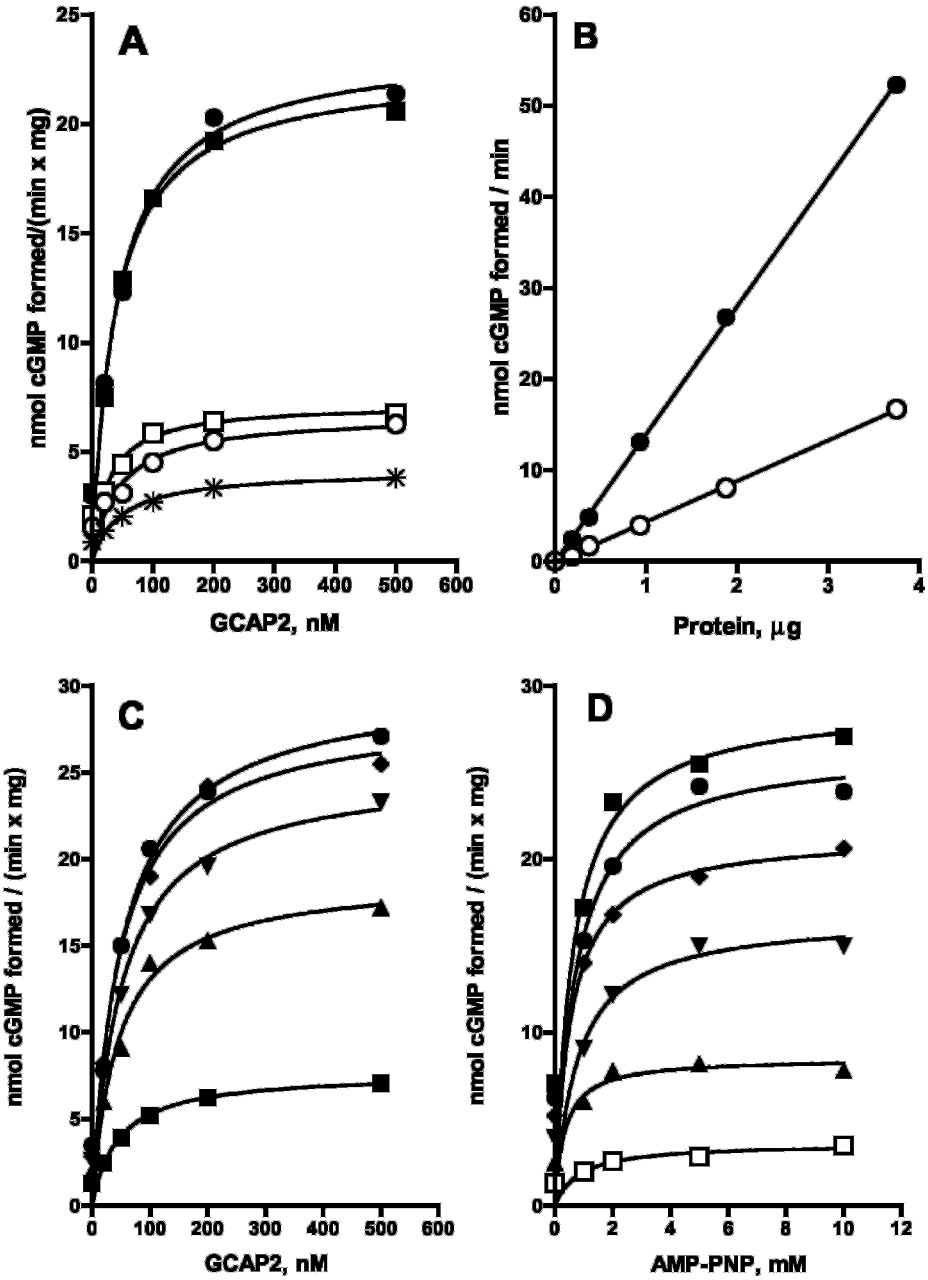

FIG. 2. **GCAP2-stimulated retGC activity after preincubation of OS homogenates with AMP-PNP.** *A*, the effect of AMP-PNP preincubation of OS homogenates on the GCAP2-stimulated retGC activity. OS homogenates (150 μg/300 μl) were preincubated with (● and ■) or without (○ and □) 5 mM AMP-PNP in the presence of high (● and ○) or low (■ and □) Ca^{2+} concentrations. For the high Ca^{2+} concentrations, 100 μM $CaCl_2$ was added; for the low concentrations, 2 mM EGTA was added. After washing out the soluble fraction, retGC activities in the membrane fraction (5.63 μg/200 μl) were assayed with indicated GCAP2 concentrations in the presence of 2 mM EGTA. As a reference, the effect of GCAP2 on the retGC activity in GCAP-free membranes (5 μg/200 μl) was also measured under the same conditions but without preincubation (*). *B*, the relationship between retGC activity and proteins in membranes after AMP-PNP preincubation. After incubation of OS homogenate (150 μg/300 μl) with (●) or without (○) 5 mM AMP-PNP, retGC activities in the various amounts of membrane fraction were measured with 200 nM GCAP2 in the assay buffer (100 μl) containing 2 mM EGTA. *C*, GCAP2 concentrations required for the large activation of retGC after preincubation of OS homogenates. After incubation of OS homogenates (150 μg/300 μl) with indicated AMP-PNP concentrations, the GCAP2-stimulated activities of retGC (5.63 μg/200 μl) were measured with indicated GCAP2 concentrations in the presence of 2 mM EGTA. The following are the AMP-PNP concentrations used: ■, 0 mM; ▲, 1 mM; ▼, 2 mM; and ♦, 5 mM; and ●, 10 mM. *D*, AMP-PNP concentrations in the preincubation of OS homogenates. The data shown in *C* were replotted to determine the GCAP2 concentration required for the large activation of retGC. The following are the GCAP2 concentrations used: □, 0 nM; ▲, 20 nM; ▼, 50 nM; ♦, 100 nM; ●, 200 nM; and ■, 500 nM.

GCAP-free membranes approximately four times under our assay conditions (from 0.877 to 3.83 nmol cGMP/min/mg formed) (Fig. 2*A*). Because the GCAP stimulation has been measured using GCAP-free membranes without preincubation with adenine nucleotides, our results indicate that most, if not all, activities of retGC/membranes described in previous studies represent only a small portion of the potential retGC activity in photoreceptors. We note that the control activity of retGC preincubated without AMP-PNP is used to express the level of retGC activation by the AMP-PNP preincubation. This comparison may be suitable to compare the activation of retGC preincubated with AMP-PNP with that preincubated without AMP-PNP and to compare the activity of retGC preincubated with AMP-PNP with the activities reported previously that were measured without preincubation with adenine nucleotides.

In AMP-PNP-pretreated OS homogenates, the retGC activity was enhanced in a GCAP2 concentration-dependent manner (Fig. 2*C*). The GCAP2 concentration required for 50% enhancement appears to be similar at all AMP-PNP concentrations used (mean for five concentrations = 47.9 ± 2.7 nM). These results indicate that the AMP-PNP preincubation increases the V_{max} of GCAP2-stimulated retGC activity but does not alter the affinity of retGC for GCAP2. We note that the real EC_{50} may be slightly higher than the average concentration because the membranes already contain some endogenous GCAP2, as described below. Generally the EC_{50} obtained here was similar to those reported previously (5, 59). The AMP-PNP concentration required for the 50% enhancement is 0.65 ± 0.20 mM (Fig. 2*D*). The maximum stimulation was achieved by ~5 mM AMP-PNP, and the stimulation was not changed even after the preincubation with 10 mM AMP-PNP.

The effect of Ca^{2+} on the AMP-PNP preincubation was also investigated (Fig. 2*A*). Without AMP-PNP, the activity of retGC preincubated under high Ca^{2+} concentrations was consistently lower than that preincubated under low Ca^{2+} concentrations (2 mM EGTA) (1.56–2.10 nmol cGMP/min/mg formed). With AMP-PNP, but without exogenous GCAP2, OS homogenates preincubated in low Ca^{2+} concentrations also show slightly higher retGC activity than that in high Ca^{2+} concentrations (3.15–2.99 nmol cGMP/min/mg formed). However,

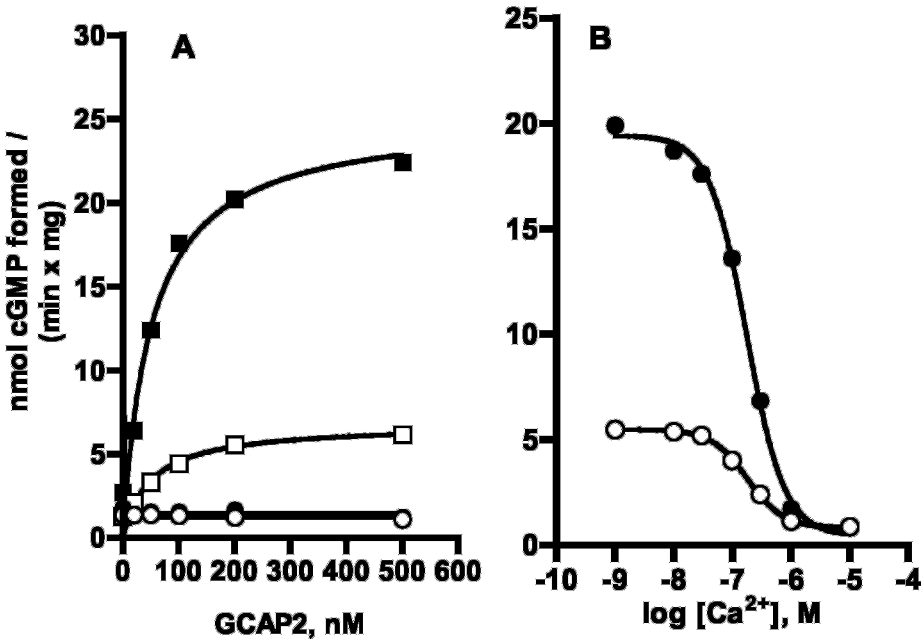

FIG. 3. **Sensitivity of GCAP2-stimulated retGC activity to high Ca^{2+} concentrations after preincubation of OS homogenates with AMP-PNP.** *A*, retGC activity measured with various amounts of GCAP2 in the presence or absence of high Ca^{2+} concentrations. OS homogenates (300 μg/600 μl) were preincubated with (■ and ●) or without (□ and ○) 5 mM AMP-PNP and membrane fractions were washed as described. RetGC activity in the membrane fractions (5.63 μg/200 μl) was measured with various amounts of GCAP2 in the presence of 2 mM EGTA (■ and □) or 100 μM $CaCl_2$ (● and ○). *B*, GCAP2-activated retGC activities measured with various Ca^{2+} concentrations. OS homogenates (150 μg/300 μl) were preincubated with (●) or without (○) 5 mM AMP-PNP, and the membrane fractions were washed. retGC activity in the membrane fractions (5.63 μg/200 μl) was measured with 200 nM GCAP2 in the presence of various Ca^{2+} concentrations. The Ca^{2+} concentrations were adjusted using Ca^{2+}-EGTA buffers.

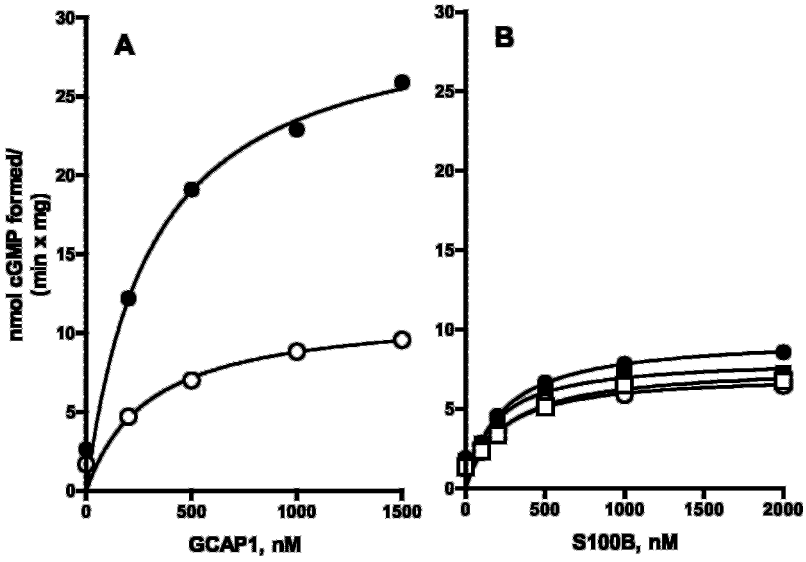

FIG. 4. **Stimulation of retGC by GCAP1 or S100B after incubation of OS homogenates with AMP-PNP.** *A*, stimulation of retGC by GCAP1. OS homogenates (150 μg/300 μl) were preincubated with (●) or without (○) 5 mM AMP-PNP, and the soluble fraction was washed out. RetGC activities in the membrane fraction (2.82 μg/100 μl) were measured with indicated amounts of GCAP1 in the presence of 2 mM EGTA. *B*, stimulation of retGC by S100B. OS homogenates (150 μg/300 μl) were pretreated with (● and ■) or without (○ and □) 5 mM AMP-PNP in the presence of 100 μM $CaCl_2$ (● and ○) or 2 mM EGTA (■ and □), and the soluble fraction was washed out. retGC activities of the membrane fraction (5.63 μg/200 μl) were measured with indicated amounts of S100B in the presence of 200 μM $CaCl_2$.

with GCAP2, the maximum activity of retGC preincubated in high Ca^{2+} concentrations was slightly higher than that in low Ca^{2+} concentrations (21.4–20.6 nmol cGMP/min/mg formed in the presence of 500 nM GCAP2). These Ca^{2+} effects were measured five times, and the observations were consistent. The higher retGC activity with the preincubation in the presence of high Ca^{2+} concentrations was more clearly observed in the ATP preincubation (Fig. 1). Although the physiological significance of the slight increase in retGC activity by the incubation in the presence of high Ca^{2+} concentrations is not clear now, these results indicate that the reduction of Ca^{2+} concentrations, essential for the retGC activation by GCAPs, is not required during the AMP-PNP preincubation.

We also checked the effect of Mg^{2+} concentrations in the preincubation on the subsequent activation of retGC by GCAP2. The preincubation was also carried out with 5 mM AMP-PNP. We found that the preincubation with 10 mM $MgCl_2$ most effectively enhanced the retGC activity. However, the differences with varying Mg^{2+} concentrations were small. For example, the activity of retGC preincubated in 10 mM $MgCl_2$ was ~110 and ~105% of those preincubated in 5 and 15 mM $MgCl_2$, respectively. Thus, throughout this study we used 5 mM $MgCl_2$ as a source of Mg^{2+} ion in the preincubation.

RetGC Stimulation by GCAP2 after Preincubation of OS Homogenates with AMP-PNP Is Sensitive to High Ca^{2+} Concentrations—The activation of retGC by GCAP2 in OS homogenates preincubated with AMP-PNP was abolished by high Ca^{2+} concentrations in the assay, and this Ca^{2+} inhibition was consistently observed regardless of GCAP2 concentrations (Fig. 3*A*). The sensitivity to high Ca^{2+} concentrations was the same as that observed in membranes preincubated without AMP-PNP (Fig. 3*B*). In the preincubation without AMP-PNP, the IC_{50} for calcium inhibition was 180 nM, and the Hill coefficient was 1.42; with AMP-PNP, the IC_{50} was 178 nM, and the Hill coefficient was 1.36. The sensitivity to high Ca^{2+} concentrations was also the same as that observed in GCAP2-stimulated retGC in GCAP-free membranes (data not shown). These results indicate that the Ca^{2+} sensitivity of GCAP-stimulated retGC is preserved after OS homogenates were preincubated with AMP-PNP and that the highly activated retGC can be regulated by change in physiological Ca^{2+} concentrations in OS.

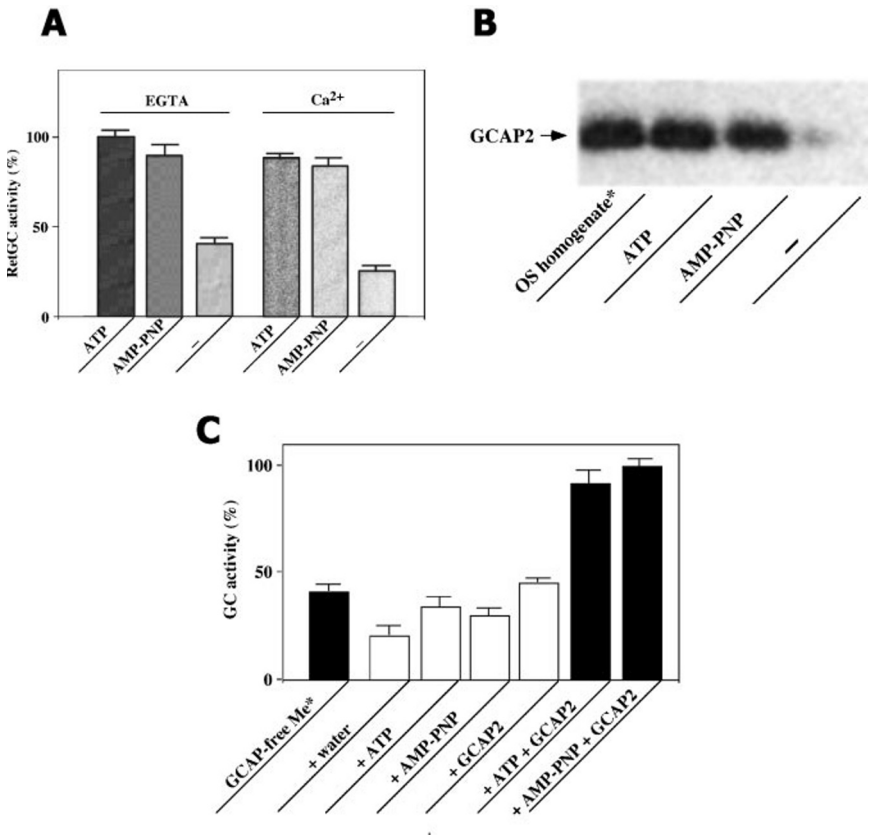

FIG. 5. **AMP-PNP preincubation enhances binding of GCAP2 to membranes containing retGC.** *A*, effect of preincubation with adenine nucleotides on retGC activities. OS homogenates (50 μg/100 μl) were preincubated with or without adenine nucleotides in the presence (100 μM) or absence (1 mM EGTA) of Ca^{2+}, and the soluble fraction was washed out. The adenine nucleotides used were ATP (1 mM) and AMP-PNP (1 mM). RetGC activities in the membrane fraction (5 μg/200 μl) were measured in the presence of 1 mM EGTA. GCAPs were not added exogenously. 100% activity indicates 3.2 nmol of cGMP/min/mg formed. *B*, detection of GCAP2 in membranes preincubated with or without adenine nucleotides. OS homogenates (50 μg/100 μl) were incubated with or without adenine nucleotides (1 mM), and then the soluble fractions were washed out. The membrane fractions were suspended in SDS sample buffer and applied to a gel for SDS-PAGE. After blotting to a polyvinylidene difluoride membrane, GCAP2 was detected using a GCAP2-specific antibody and a chemiluminescent substrate. Control (*OS homogenate**) indicates the original amounts of GCAP2 in OS homogenates (50 μg). *C*, effect of preincubation of GCAP-free membranes with various combination of GCAP2 and adenine nucleotides on retGC activity. GCAP-free membranes (20 μg/40 μl) were incubated with indicated combinations of adenine nucleotides (1 mM) and GCAP2 (2.5 μM). After washing out soluble components, retGC activities in membranes (5 μg/200 μl) were measured in the presence of 1 mM EGTA. 100% activity indicates 4.2 nmol of cGMP/min/mg formed.

GCAP1, but Not S100B, Also Enhances retGC Activity after Preincubation of OS Homogenates with AMP-PNP—After preincubation of OS homogenates with or without AMP-PNP, GCAP1-stimulated retGC activity was measured (Fig. 4*A*). Without AMP-PNP, the retGC activity was stimulated by GCAP1 (1.5 μM) approximately six times (from 1.69 to 9.57 nmol cGMP/min/mg formed), whereas with AMP-PNP (5 mM), the GCAP1-stimulated retGC activity was about 15 times of the control activity (from 1.69 to 25.9 nmol cGMP/min/mg formed). The retGC control activity was also increased by the AMP-PNP preincubation (from 1.69 to 2.62 nmol cGMP/min/mg formed). As was the case with GCAP2, the concentration for half-maximal stimulation by GCAP1 was similar in the absence (EC_{50} = 298 nM) or presence (EC_{50} = 308 nM) of AMP-PNP in the preincubation. The EC_{50} was similar to that reported previously (43). However, when retGC in these membranes was activated by S100B, the AMP-PNP preincubation only negligibly increased the retGC activity (Fig. 4*B*). In the experiments, the AMP-PNP preincubation was carried out with high or low Ca^{2+} concentrations (100 μM $CaCl_2$ or 2 mM EGTA), indicating that the high activation does not occur regardless of Ca^{2+} concentrations in the preincubation. Together, observations shown here suggest that the large stimulation of retGC in OS homogenates preincubated with AMP-PNP occurs only when retGC is stimulated by GCAPs.

Endogenous GCAPs Remain Bound to retGC/Membranes during Washing after the AMP-PNP Preincubation—As described above, the AMP-PNP preincubation is critical for the subsequent large activation of retGC by GCAPs. Thus, we have closely studied retGC activities with various preincubation conditions. We found that the retGC activity in OS homogenates preincubated with adenine nucleotides (1 mM) was approximately two times those preincubated without adenine nucleotides (Fig. 5*A*). The high activities were detected regardless of Ca^{2+} concentrations in the preincubation, although preincubation with a low Ca^{2+} concentration (1 mM EGTA) showed slightly higher retGC activities than those with a high Ca^{2+} concentration. We also found that these activities were sensitive to high Ca^{2+} concentrations (data not shown). These results indicate that endogenous GCAPs remain bound to retGC/membranes during washing and function as retGC activators in the adenine nucleotide preincubation. We note that these results are similar to those observed in the preincubation with 5 mM AMP-PNP (Fig. 2), suggesting that similar protein-protein interactions occur in the preincubation. Experiments using a GCAP2-specific antibody (Fig. 5*B*) support this notion. We detected GCAP2 in membranes after washing only when OS homogenates were preincubated with adenine nucleotides. The result apparently indicates that much of endogenous GCAP2 was associated with retGC/membranes. The reduction of retGC activity by high Ca^{2+} concentrations (Fig. 5*A*) may be due to the

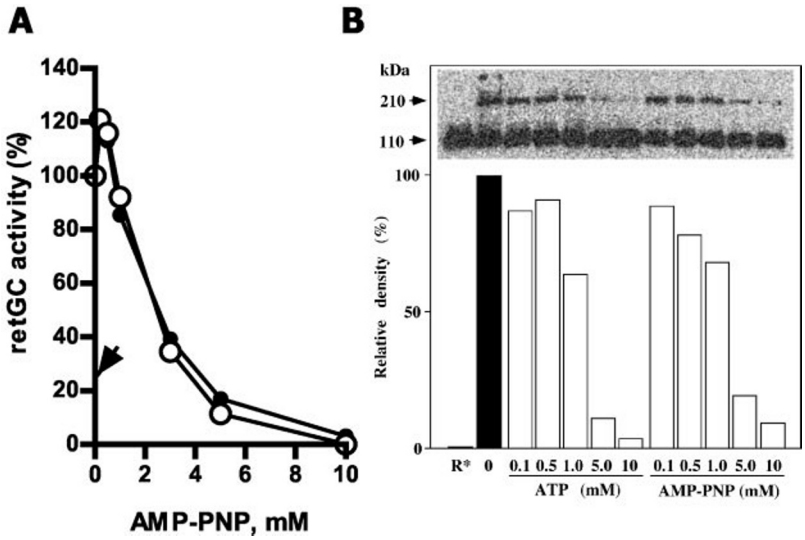

FIG. 6. **Effects of AMP-PNP on retGC during the preincubation of OS homogenates.** *A*, inhibition of retGC by high AMP-PNP concentrations. retGC activities in OS homogenates (5 μg/200 μl) were measured with indicated AMP-PNP concentrations in the presence of 5 (○) or 20 (●) mM $MgCl_2$. The retGC activities were stimulated by the addition of 2 mM EGTA. 100% indicates 5.78 (5 mM $MgCl_2$) and 5.57 (20 mM $MgCl_2$) nmol of cGMP/min/mg formed. The *arrow* indicates the retGC activity measured in the presence of 100 μM Ca^{2+}. *B*, effect of AMP-PNP on retGC dimerization. OS homogenates (50 μg) were incubated with indicated amounts of adenine nucleotides in the presence of 1 mM EGTA, and retGC dimers formed were cross-linked using bis(sulfosuccinimidyl) suberate as described previously (25). After separation of retGC dimer (210 kDa) from its monomeric form (110 kDa) by SDS-PAGE and blotting to a polyvinylidene difluoride membrane, these retGC proteins were detected with a retGC-1-specific antibody and a chemiluminescent substrate. The bands were scanned and the relative density ($mm^2 \times OD$) was calculated. To compare the level of dimerization with retGC activities, the dimerization was performed in the presence of 1 mM GTP and 1 mM cGMP. *R** indicates retGC without bis(sulfosuccinimidyl) suberate. The *upper panel* is the retGC dimer detected in the polyvinylidene difluoride membrane, and the *lower panel* is the relative density of the 210-kDa band.

weaker binding of GCAP2 to membranes (and/or to retGC) in the presence of high Ca^{2+} (60).

To further test this notion, experiments were carried out using GCAP-free membranes reconstituted with exogenous GCAP2 in the presence or absence of adenine nucleotides (Fig. 5*C*). We found that only membranes pretreated with adenine nucleotides in combination with GCAP2 had higher retGC activities. A previous study showing that ATP (0.4 mM) increased the efficacy of GCAP1 to stimulate retGC-1 (43) suggests that GCAP1 also shows the similar interaction with retGC in the presence of adenine nucleotides. The simplest explanation of these phenomena is that GCAP-binding sites directly connected with retGC activation in membranes were increased by the incubation with adenine nucleotide and more endogenous GCAPs bound to these sites. In the preincubation, washing conditions used were not enough to exclude all GCAPs bound. Another explanation may be that after the incubation with adenine nucleotides, endogenous GCAPs became tightly associated with retGC/membranes, remained bound to the membranes during washing, and functioned as a retGC activator. However, the possibility of this explanation may not be high because the retGC affinity to GCAP2 was not changed by the preincubation with or without adenine nucleotides (Fig. 2*C*).

RetGC Is Present as a Monomer during the Preincubation with Adenine Nucleotides—In our previous study, we suggested using a cross-linker that dimerization of retGC is essential for the activation of retGC by GCAPs (25). Here, using the same cross-linking procedure, we investigated whether the retGC dimers are formed by endogenous GCAPs during the preincubation. Because the preincubation, with high or low Ca^{2+} concentrations, enhances the GCAP-activation of retGC (Figs. 1 and 2*A*), we examine conditions with high or low Ca^{2+} concentrations. In the presence of low Ca^{2+} concentrations (2 mM EGTA), relatively high activity of retGC was detected because of the stimulation of retGC by endogenous GCAPs (Fig. 6*A*). However, the activity was inhibited by AMP-PNP in a concentration-dependent manner, and 5 mM AMP-PNP, the AMP-PNP concentration used to preincubate OS homogenates, suppressed ~85% of the activity. The inhibition was similar in the presence of low (5 mM) or high (20 mM) $MgCl_2$, indicating that the inhibition is not due to the shortage of Mg^{2+}. We also found that high concentrations of adenine nucleotides inhibited the formation of a 210-kDa retGC complex (Fig. 6*B*). The retGC complex has been shown to be a homodimer of retGC (25). The inhibition of the retGC complex formation appears to be parallel to the reduction of retGC activity, strongly suggesting that the inhibition of retGC dimerization is involved in the retGC inhibition by adenine nucleotides, although we do not have any data to exclude the possibility that the adenine nucleotide inhibition is due to binding of adenine nucleotides at the catalytic site. In the presence of high Ca^{2+} concentrations, retGC in OS homogenates preincubated with AMP-PNP was inactive (Fig. 3*A*), and retGC exists as a monomeric form even in the absence of AMP-PNP (25). Together, these observations indicate that retGC exists as a monomer during preincubation of OS homogenates with adenine nucleotides regardless of Ca^{2+} concentrations.

Low AMP-PNP concentrations slightly enhanced the retGC activity in OS homogenates (Fig. 6*A*). This is consistent with data reported previously (38–41). Is the enhancement due to the stimulation of retGC dimerization by low adenine nucleotide concentrations? The result (Fig. 6*B*) suggests that the level of retGC dimerization appears not to be affected by low adenine nucleotide concentrations. We did the same experiment five times; however, the results were not consistent. The lack of reproducibility suggests that clear evidence for the effect of low adenine nucleotide concentrations on retGC dimerization may be difficult to obtain, in part because the effect is small. In addition, concentrations of adenine nucleotides used were less than the physiological concentration of ATP (3–4 mM). Moreover, it is doubtful that the ATP concentration in ROS becomes so low under normal conditions, because the average ATP concentration is not changed by light, as reported previously

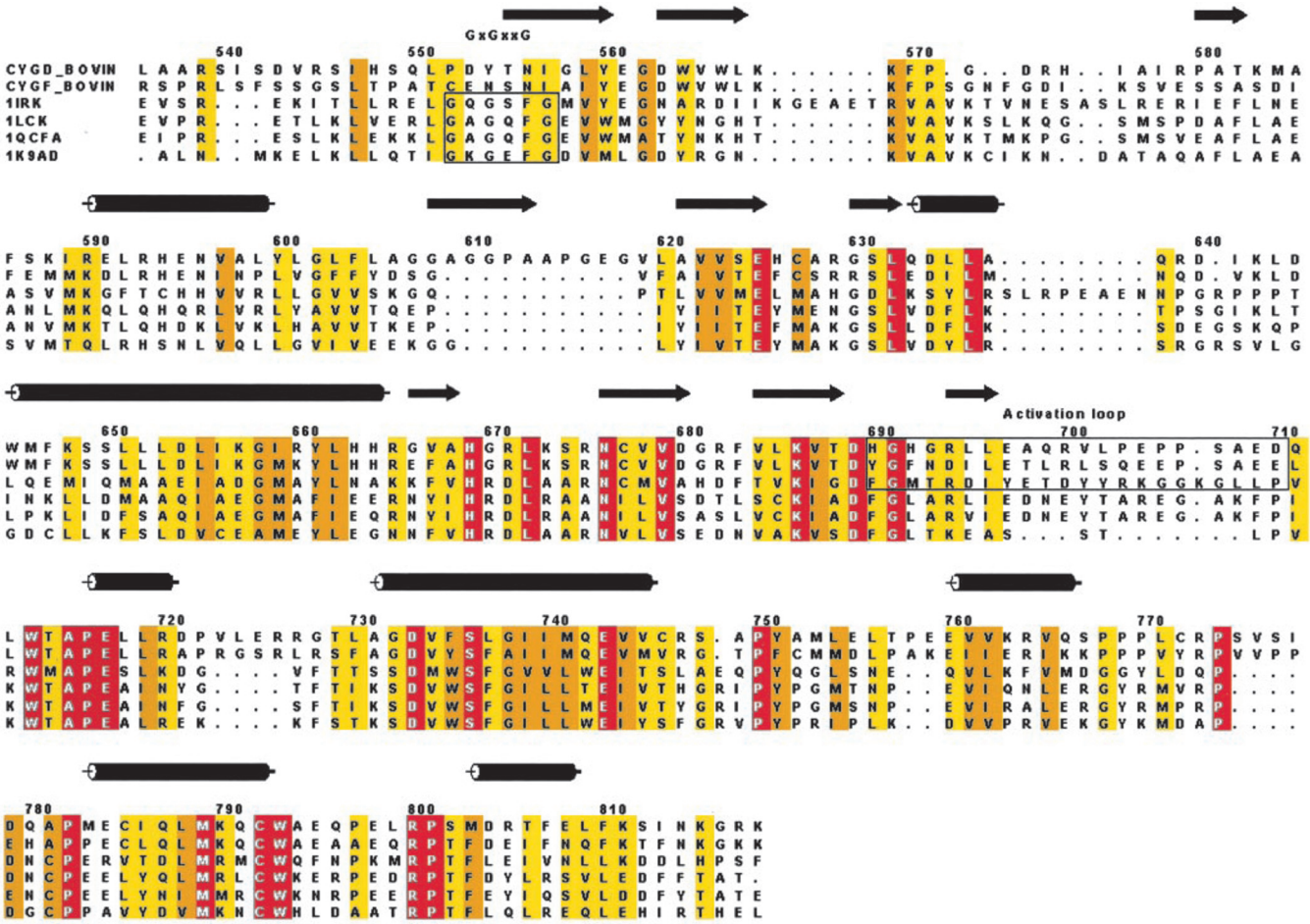

FIG. 7. **Alignment of bovine retGC KHD with the ATP-binding sites of the insulin receptor and other kinases.** A multiple sequence alignment of retGC-1 (CYGD_BOVIN) and retGC-2 (CYGF_BOVIN) with the human insulin receptor kinase domain (1IRK) and other protein kinase domains was used to define the kinase homology domains of retGC-1 and -2. The Protein Data Bank Code (modified in some cases by Swiss Model for single polypeptide chains) and the kinase identification are as follows: 1LCK, human p56-LCK tyrosine kinase; 1QCFA, hematopoietic cell kinase; 1K9AD, carboxyl-terminal Src kinase. The program ALSCRIPT (72) was used to display the alignment. Sequence identities are shown in *red*, conservative substitutions are indicated in *orange*, and semi-conservative substitutions are in *yellow*. The sequence numbers refer to the CYGD_BOVIN (retGC-1) sequence. The *arrows* above the sequences indicate regions of β strand; *cylindrical symbols* indicate α helical regions as predicted in the model of the AMP-PNP-bound form of retGC-1. The boxed area 552–557 indicates the G*X*G*XX*G motif necessary for kinases. The boxed region 690–710 indicates the insulin receptor activation loop and the putative activation loops of retGC-1 and -2.

(61). Thus, the significance of the activation of retGC by low concentrations of adenine nucleotides is unclear.

DISCUSSION

This study has been carried out to obtain answers to a fundamental but hitherto unexplored question regarding the regulation of retGC. The question is whether the GCAP stimulation of retGC previously observed can be further enhanced. As an answer, we found that retGC could be activated by GCAPs at least 10–13-fold over the control activity and that interaction with adenine nucleotides was essential for this high activation of retGC. Significantly, recent studies using a double knockout mouse ($GCAP^{-/-}$) showed that retGC is activated *in vivo* by GCAPs ~13-fold over the control activity, and the level of stimulated retGC activity is similar or higher than that of light-activated PDE (34, 35, 49). Our observations are in good agreement with those *in vivo* studies. We feel that the large stimulation of retGC reported here represents a major portion of the mechanism involved in the retGC activation detected *in vivo*.

In the previous model, the retGC activation by GCAPs is a one-step mechanism: activation by GCAPs when the Ca^{2+} concentration in OS is reduced. Low concentrations (less than 0.5 mM) of adenine nucleotides in the assay mixture were shown to stimulate retGC activity; however, the activation was generally small in OS membranes (less than 1.5 times) (Refs. 21 and 38–43 and Fig. 6*A*). Thus, adenine nucleotides were believed to modify the activity but not to be essential. Most aspects, if not all, of retGC including its physiological roles, its regulations, and its function-structure relationships appear to be interpreted based on the one-step mechanism. Here, based on the results described, we propose a two-step mechanism for the retGC activation: retGC first interacts with ATP to produce a conformational change and is then highly activated by GCAPs when the Ca^{2+} concentration is reduced. As described above, we estimated that less than 20 μM AMP-PNP were carried over from the preincubation mixture to the retGC preparation. This indicates that less than 1 μM AMP-PNP presented in the assay mixture because 10 μl of the retGC preparation was added to assay mixture (200 μl). This AMP-PNP concentration was too small to activate retGC in the assay mixture (Fig. 6*A*). Under the conditions, GCAPs stimulated retGC (Fig. 2). Thus, in this mechanism, the step for the ATP interaction and the step for the GCAP activation are separate and distinct, and both steps are essential for the large stimulation. This adenine nucleotide interaction requires concentrations in the millimolar range (EC_{50} = 0.65 mM for AMP-PNP) along with other factor(s)

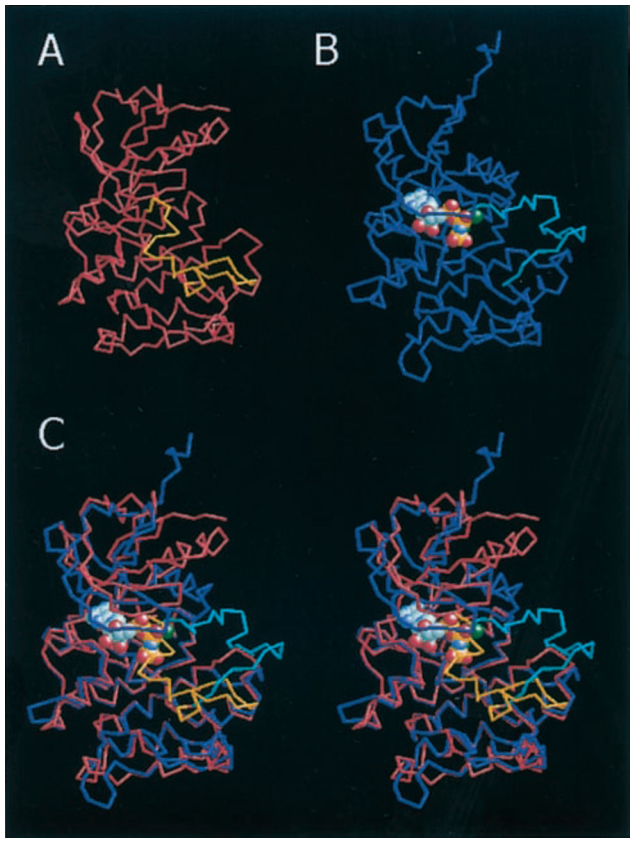

FIG. 8. **Models of bovine retGC-1 KHD in the absence and presence of AMP-PNP.** The multiple sequence alignment of retGC-1 with the insulin receptor kinase (1IRK) was used as a guide for modeling with the Swiss PdbViewer program in conjunction with the online Swiss Model program. The putative inactive conformation of retGC-1 was modeled using the coordinates of the inactive insulin receptor kinase (Protein Data Bank code 1IRK). The AMP-PNP-activated form was modeled using the coordinates of Protein Data Bank code 1IR3. The images were generated using RasMol. *A* shows the inactive conformation of retGC-1 with the activation loop shown in *orange*. *B* shows the AMP-PNP-bound conformation in the same orientation with the activation loop shown in *cyan*. *C* shows a stereo view of the superposition of the two conformations.

(discussed below) and is indifferent to the calcium concentration. On the other hand, the adenine nucleotide stimulation in the assay mixtures requires adenine nucleotide concentrations below the millimolar range because higher concentrations inhibit retGC activity either directly or through inhibition of dimerization. In addition, the stimulation in the assay mixtures requires low calcium concentrations for the activation by GCAPs.

Is the mechanism for retGC activation by the adenine nucleotide preincubation different from the activation by low concentrations of adenine nucleotides in the assay mixture? The retGC activity in AMP-PNP-preincubated OS membranes was further activated by the low concentrations of AMP-PNP in the assay mixture.[2] This implies that the AMP-PNP in the assay mixture further activates retGC already stimulated by the AMP-PNP preincubation and that the AMP-PNP activation in the assay mixture does not require the unknown factor(s) (discussed below) required for the activation by AMP-PNP preincubation. These implications are suggestive of the mechanistic difference but not conclusive. On the other hand, in some studies retGC activities similar to those stimulated by AMP-PNP preincubation were reported without the adenine nucleotide preincubation (58, 60). These activities were measured with low concentrations (<0.5 mM) of ATP in the assay mixture. Although these high retGC activities were not constantly observed even in the same research groups and the ATP contribution to the high activities is unknown, these activities may suggest that under certain conditions a mechanism similar to that for the activation by adenine nucleotide preincubation may function in the assay mixture.

Using AMP-PNP, we suggest that binding of ATP to retGC produces the large activation by facilitating subsequent interaction with GCAPs. However, it should be emphasized that binding of ATP to retGC is speculative based on the observation that preincubation of OS homogenates with AMP-PNP was required for high activation of retGC. However, this speculation is also supported by previous studies as follows. (*a*) RetGC has a molecular configuration similar to those of peptide-regulated GCs (17, 18, 37), implying that the regulatory mechanism of retGC may be similar to those of peptide-regulated GCs. In peptide-regulated GCs, ATP is an important factor for their regulation (17, 18, 37). It has been hypothesized in these GCs that ATP binds to the KHD, serves as an intracellular allosteric regulator, and releases an inhibitory constraint of the KHD for the activation. (*b*) A previous study already suggested that AMP-PNP binds to retGC (58). Although the study did not directly show the AMP-PNP binding to retGC, the AMP-PNP binding appears to be a reasonable explanation. c) As discussed above, activation of retGC by low concentrations of ATP and its analogs has been reported in previous studies (38–41), although the concentration of adenine nucleotides is different from those in this study. This stimulation has been speculated to be due to the binding of ATP to the KHD in retGC.

To test the possibility that adenine nucleotides function as allosteric regulators by binding to the KHD, we used three-dimensional modeling. The availability of crystal structures for the inactive and active conformations of the insulin receptor kinase domain (62) allowed construction of models for the KHD of retGC with and without bound AMP-PNP. For this purpose, an alignment of bovine retGC-1 with the ATP binding sites of the insulin receptor and other kinases was carried out to determine the boundaries of the KHD within the retGC-1 sequence. Fig. 7 displays the alignment that indicates sequence similarity allowing three-dimensional modeling. Also indicated is the lack of the G*X*G*XX*G motif (the boxed region 552–557) necessary for kinases in the retGC-1 sequence, a feature previously noted (37, 63).

In the absence of AMP-PNP binding, the KHD was modeled in a conformation corresponding to the inactive insulin receptor kinase domain. Fig. 8*A* shows the model generated for the AMP-PNP-free form of retGC-1 with a loop shown in *orange* that corresponds to the activation loop (the boxed region 690–710 of Fig. 7) of the insulin receptor. In this conformation, the putative activation loop occupies the potential adenine nucleotide-binding site. We propose that in this conformational state, GCAPs may not effectively interact with retGC to stimulate activity. Upon binding of AMP-PNP, the KHD of retGC can be modeled in a conformation corresponding to the activated insulin receptor kinase (Fig. 8*B*). The putative activation loop of retGC (shown here in *cyan*) is now exposed along with a groove corresponding to the peptide-binding site of the insulin receptor kinase, allowing new intra- and inter-protein interactions such as with GCAPs. Fig. 8*C* shows a stereo view of the superposition of the two conformations. Modeling of inactive and active conformations of retGC-2 produced similar results (data not shown).

However, it should be emphasized that this study does not exclude the possibility that protein phosphorylation also plays a role in the large activation of retGC. The preincubation of OS

[2] A. Yamazaki, unpublished observations.

homogenates with adenine nucleotides was initially carried out under the conditions for protein phosphorylation. Comparison of specific activities of retGC shown in Figs. 1 and 2 shows that ATP is approximately twice as effective as AMP-PNP for the retGC stimulation under our conditions. Moreover, phosphatase inhibitors (10 nM okadaic acid and 10 mM NaF) further increased the retGC activity.[2] These results suggest that phosphorylation of retGC and/or proteins involved in the retGC regulation may further enhance the GCAP-dependent retGC activity that has already been stimulated by binding of adenine nucleotides. For retGC, we have already detected incorporation of the γ-phosphate of ATP into the protein under the conditions used for preincubation.[3] Previous studies also reported that retGC might be regulated by phosphorylation (64–66). Moreover, it has been reported that phosphorylation, in addition to ATP binding, is important for the activation of GC-A (18, 67). For proteins involved in the retGC regulation, GCAPs are the established candidates, although phosphorylation of an unknown protein regulator(s) (discussed below) may also be possible. It has been reported that GCAP2 is phosphorylated by cyclic nucleotide-dependent protein kinases, but the phosphorylation had little effect on retGC activation by GCAP2 (68). The study appears to have been conducted under conditions for the single-step mechanism. It may not be surprising to detect that GCAP phosphorylation is involved in the retGC regulation under the two-step mechanism reported here. We also note that the GCAP2-stimulated activity of retGC preincubated in the presence of high Ca^{2+} concentrations was slightly but consistently higher than that in the presence of low Ca^{2+} concentrations when AMP-PNP was added to the preincubation mixture (Fig. 2*A*). It is possible that the difference found in the AMP-PNP preincubation may be due to protein dephosphorylation because phosphatase inhibitors were not in the preincubation mixture. This Ca^{2+} effect was detected more clearly when ATP was used (37 °C) (Fig. 1). These observations imply that if protein phosphorylation is involved in the large activation, the phosphorylation level may be Ca^{2+}-regulated.

Because this study is the first report for the two-step mechanism, new questions have arisen that will require answers. We found that addition of GCAP2 to prewashed membranes followed by the incubation with AMP-PNP was not enough to obtain the large activation.[2] These observations suggest that the simple binding of AMP-PNP to retGC with GCAP2 was not enough for the high activation. The possibility that AMP-PNP cannot bind to retGC in washed membranes may be excluded by a previous study suggesting that AMP-PNP appears to change the retGC structure in washed membranes (58). The simplest explanation to our observations is that a factor(s) in OS homogenates may be required in the AMP-PNP preincubation.

Another puzzling point is that the large activation was detected even after washing out of soluble components from OS homogenates. This indicates that the effect of AMP-PNP is retained on retGC after the washing, although EC_{50} of AMP-PNP for the large activation is relatively high (0.64 mM). The mechanism to retain the effect of AMP-PNP on retGC is unclear. We speculate that after interaction with AMP-PNP, retGC changes its conformation, and the conformational change prevents AMP-PNP from being released from retGC. Alternatively, AMP-PNP may be released from retGC during the washing, but the conformational change in retGC is retained by a factor(s) in OS homogenates. Although there may be other explanations, it is crucial to elucidate all of the components in OS homogenates involved for understanding of the large activation of retGC.

[3] H. Yu and A. Yamazaki, unpublished observations.

We have shown that endogenous GCAP2 is associated with retGC/membranes after preincubation of OS homogenates with adenine nucleotides (Fig. 5) and that the large activation of the retGC is accomplished by addition of exogenous GCAP2 (Fig. 2). How many GCAP-binding sites are on the adenine nucleotide-treated retGC? As a model for one binding site, we presume two retGC conformations in equilibrium: an inactive form without enzyme activity and possessing no ability to be stimulated by GCAP2 and an active form with enzyme activity that is dependent on GCAP2. Thus, the observed activity is dependent upon the proportion of the active form and the content of GCAP2. Without adenine nucleotide treatment, only a small fraction of retGC exists as the active form. The small increase of the retGC activity by exogenous GCAP2 would be due to that portion of the active form whose GCAP2 sites are not already filled by endogenous GCAP2, *i.e.* exogenous GCAP2 binds to the residual GCAP-free active form to express retGC activity. However, because the fraction of total retGC present as active form is small, the observed retGC activity is not large (Fig. 2). The requirement for exogenous GCAP2 may be due to low content of GCAP2 compared with retGC in the preparations used and/or the loss by washing. This explanation can also be applied to the basal activity and GCAP-dependent activation of retGC in previous studies. Treatment with adenine nucleotides shifts the equilibrium from the inactive form toward the active form. The large activation by adenine nucleotide preincubation (Fig. 2) is completed by binding of exogenous GCAP2 to the resulting larger fraction of retGC in the active form. This model is supported by the observations that the affinity of retGC for GCAP2 is not changed by preincubation with or without AMP-PNP (Fig. 2*C*) and that the level of retGC activity observed with saturating amounts of GCAP2 is determined by the concentration of AMP-PNP in the preincubation (Fig. 2*D*).

Other explanations including multiple GCAP2-binding sites are also possible. The GCAP2 added exogenously may bind to a retGC domain(s) different from the domain with which endogenous GCAP2 is associated. It is possible that the new conformation of retGC, a product of adenine nucleotide binding to retGC, opens a new binding site(s) for GCAP2. We believe that GCAP1 also associates with retGC under the same conditions, as described above. If so, which GCAP combinations can more effectively activate retGC? This question may lead not only to an explanation for the presence of two GCAPs, 1 and 2, in ROS (30, 31) but also to a new mechanism of retGC regulation.

It should be emphasized that a new preincubation of OS homogenates with adenine nucleotides is required for the large activation of retGC, although the adenine nucleotide effect is retained after washing of membranes. This implies that the adenine nucleotide effect is initiated and then terminated under certain conditions, *i.e.* there are mechanisms to turn on and turn off the ATP effect in OS. In particular, the mechanism to turn off the highly activated retGC may be as important as turning off light/GTP-activated PDE in the overall control of cGMP metabolism in OS during normal function and adaptation. Several mechanisms for the inhibition of retGC have been reported (69, 70), although these inhibitions cannot be easily incorporated in the current model of retGC regulation: the single-step mechanism. Under the new mechanism of retGC regulation reported here, these retGC inhibitory mechanisms may be incorporated more easily. We especially emphasize that retGC inhibition by RGS9 (70, 71) is attractive because under the large activation of retGC, the mutual regulation between the retGC system and the PDE system is more important, and RGS9 has been proposed to function in both the PDE and retGC systems. Obviously, further studies are needed to establish the mechanism suggested here and to answer puzzling

points. Moreover, it is important to reveal mechanisms to overcome the retGC inhibition by physiological ATP concentrations in the system proposed here. It is clear, however, that this study opens a new field of retGC regulation in photoreceptor OS.

Acknowledgments—We thank Drs. R. B. Needleman (Wayne State University) and A. Sitaramayya (Oakland University, Rochester, Michigan) for critical reading of the manuscript.

REFERENCES

1. Stryer, L. (1986) *Annu. Rev. Neurosci.* **9,** 87–119
2. Miller, W. H. (1990) *Invest. Ophthalmol. Visual Sci.* **31,** 1664–1673
3. Pugh, Jr. E. N., and Lamb, T. D. (1993) *Biochim. Biophys. Acta* **1141,** 111–149
4. Yau, K.-W., and Baylor, D. A. (1989) *Annu. Rev. Neurosci.* **12,** 289–327
5. Dizhoor, A. M., and Hurley, J. B. (1999) *Methods* **19,** 521–531
6. Fain, G. L., Matthews, H. R., Cornwall, C., and Koutalos, Y. (2001) *Physiol. Rev.* **81,** 117–151
7. Lolley, R. N., and Racz, E. (1982) *Vision Res.* **22,** 1481–1486
8. Koch, K.-W., and Stryer, L. (1988) *Nature* **334,** 64–66
9. Shyjan, A. W., de Sauvage, F. J., Gillett, N. A., Goeddel, D. V., and Lowe, D. G. (1992) *Neuron* **9,** 727–737
10. Goraczniak, R. M., Duda, T., Sitaramayya, A., and Sharma, R. K. (1994) *Biochem. J.* **302,** 455–461
11. Yang, R.-B., Foster, D. C., Garbers, D. L., and Fulle, H.-J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 602–606
12. Lowe, D. G., Dizhoor, A. M., Liu, K., Gu, Q., Spencer, M., Laura, R., Lu, L., and Hurley, J. B. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 5535–5539
13. Johnston, J. P., Farhangfar, F., Aparicio, J. G., Nam, S. H., and Applebury, M. L. (1997) *Gene* (*Amst.*)**193,** 219–227
14. Goraczniak, R. K., Duda, T., and Sharma, R. K. (1997) *Biochem. Biophys. Res. Commun.* **234,** 666–670
15. Liu, X., Seno, K., Nishizawa, Y., Hayashi, F., Yamazaki, A., Matsumoto, H., Wakabayashi, T., and Usukura, J. (1994) *Exp. Eye Res.* **59,** 761–768
16. Cooper, N., Liu, L., Yoshida, A., Ponzdnyakov, N., Margulis, A., and Sitaramayya, A. (1996) *J. Mol. Neurosci.* **6,** 211–222
17. Lucas, K., A., Pitari, G. M., Kazerounian, S., Ruiz-Stewart, I., Park, J., Schulz, S., Chepenik, K. P., and Waldman, S. A. (2000) *Pharmacol. Rev.* **52,** 375–413
18. Potter, L. R., and Hunter, T. (2001) *J. Biol. Chem.* **276,** 6057–6060
19. Gorczyca, W. A., Grey-Keller, M. P., Detwiler, P. B., and Palczewski, K. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 4014–4018
20. Dizhoor, A. M., Lowe, D. G., Olshevskaya, E. V., Laura, R. P., and Hurley, J. B. (1994) *Neuron* **12,** 1345–1352
21. Laura, R. P., Dizhoor, A. M., and Hurley, J. B. (1996) *J. Biol. Chem.* **271,** 11646–11651
22. Duda, T., Goraczniak, R., Surgucheva, I., Rudnicka-Nawrot, M., Gorczyca, W. A., Palczewski, K., Sitaramayya, A., Baehr, W., and Sharma, R. K. (1996) *Biochemistry* **35,** 8478–8482
23. Haeseleer, F., Sokal, I., Li, N., Pettenati, M., Rao, N., Bronson, W., Wechter, R., Baehr, W., and Palczewski, K. (1999) *J. Biol. Chem.* **274,** 6526–6535
24. Yang, R.-B., and Garbers, D. L. (1997) *J. Biol. Chem.* **272,** 13738–13742
25. Yu, H., Olshevskaya, E., Duda, T., Seno, K., Hayashi, F., Sharma, R. K., Dizhoor, A. M., and Yamazaki, A. (1999) *J. Biol. Chem.* **274,** 15547–15555
26. Tucker, C. L., Woodcock, S. C., Kelsell, R. E., Ramamurthy, V., Hunt, D. M., and Hurley, J. B. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 9039–9044
27. Duda, T., Venkataraman, V., Jankowska, A., Lange, C., Koch, K.-W., and Sharma, R. K. (2000) *Biochemistry* **39,** 12522–12533
28. Pozdnyakov. N., Yoshida, A., Cooper, N. G. F., Margulis, A., Duda, T., Sharma, R. K., and Sitaramayya, A. (1995) *Biochemistry* **34,** 14279–14283
29. Margulis, A., Pozdnyakov, N., and Sitaramayya, A. (1996) *Biochem. Biophys. Res. Commun.* **218,** 243–247
30. Cuenca, N., Lopez, S., Howes, K., and Kolb, H. (1998) *Invest. Ophthlmol. Visual Sci.* **39,** 1243–1250
31. Kachi, S., Nishizawa, Y., Olshevskaya, E., Yamazaki, A., Miyake, Y., Wakabayashi, T., Dizhoor, A., and Usukura, J. (1999) *Exp. Eye Res.* **68,** 465–473
32. Payne, A. M., Downes, S. M., Bessant, D. A. R., Taylor, R., Holder, G. E., Warren, M. J., Bird, A. C., and Bhattachraya, S. S. (1998) *Hum. Mol. Genet.* **7,** 273–277
33. Dizhoor, A. M., Boikov, S. G., and Olshevskaya, E. V. (1998) *J. Biol. Chem.* **273,** 17311–17314
34. Mendez, A., Burns, M. E., Sokal, I, Dizhoor, A. M., Baehr, W., Palczewski, K., Baylor, D. A., and Chen, J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 9948–9953
35. Burns, M., Mendez, A., Chen, J., and Baylor, D. A. (2002) *Neuron* **36,** 81–91
36. Howes, K. A., Pennesi, M. E., Sokal, I., Church-Kopish, J., Schmidt, B., Margolis, D., Frederick, J. M., Rieke, F., Palczewski, K., Wu, S. M., Detwiler, P. D., and Baehr, W. (2002) *EMBO J.* **21,** 1–10
37. Sharma, R. K., Yadav, P., and Duda, T. (2001) *Can. J. Physiol. Pharmacol.* **79,** 682–691
38. Krishnan, N., Fletcher, R. T., Chader, G. J., and Krishna, G. (1978) *Biochim. Biophys. Acta* **523,** 506–515
39. Sitaramayya, A., Marala, R. B., Hakki, S., and Sharma, R. K. (1991) *Biochemistry* **30,** 6742–6747
40. Gorczyca, W. A., van Hooser, P., and Palczewski, (1994) *Biochemistry* **33,** 3217–3222
41. Sitaramayya, A., Duda, T., and Sharma, R. K. (1995) *Mol. Cell. Biochem.* **148,** 139–145
42. Frins, S., Bonigk, W., Muller, F., Kellner, R., Koch, K.-W. (1996) *J. Biol. Chem.* **271,** 8022–8027
43. Otto-Bruc, A., Buczylko, J., Surguchheva, I., Subbaraya, I., Rudnicka-Nawyot, M., Crabb, J. W., Arendt, A., Hargrave, P. A., Baehr, W., and Palczewski, K. (1997) *Biochemistry* **36,** 4295–4302
44. Robinson, W. E., and Hagins, W. A. (1979) *Nature* **280,** 398–400
45. Hakki, S., and Sitaramayya, A. (1990) *Biochemistry* **29,** 1088–1094
46. Hayashi, F., and Yamazaki, A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 4746–4750
47. Koch, K.-W. (1991) *J. Biol. Chem.* **266,** 8634–8637
48. Aparicio, J. G., and Applebury, M. L. (1995) *Protein Expression Purif.* **6,** 501–511
49. Detwiler, P. D. (2002) *Neuron* **36,** 3–4
50. Cornwall, M. C., and Fain, G. L. (1994) *J. Physiol.* **480,** 261–279
51. Koutalos, Y., Nakatani, K., Tamura, T., and Yau, K.-W. (1995) *J. Gen. Physiol.* **106,** 863–890
52. Yamazaki, A., Tatsumi, M., and Bitensky, M. W. (1988) *Methods Enzymol.* **159,** 702–710
53. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. J. (1997) *Nucleic Acids Res.* **25,** 4876–4882
54. Guex, N., and Peitsch, M. C. (1997) *Electrophoresis* **18,** 2714–2723
55. Hayashi, F., Hutson, L. D., Kishigami, A., Nagao, S., and Yamazaki, A. (1993) *Methods Neurosci.* **15,** 237–247
56. Yamazaki, A., Tatsumi, M., Torney, D. C., and Bitensky, M. W. (1987) *J. Biol. Chem.* **262,** 9316–9323
57. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
58. Tucker, C. L., Laura, R. P., and Hurley, J. B. (1997) *Biochemistry* **36,** 11995–12000
59. Laura, R. P., and Hurley, J. B. (1998) *Biochemistry* **37,** 11264–11271
60. Olshevskaya, E. V., Hughes, R. E., Hurley, J. B., and Dizhoor, A. M. (1997) *J. Biol. Chem.* **272,** 14327–14333
61. Biernbaum. M. S., and Bownds, M. D. (1979) *J. Gen. Physiol.* **74,** 649–669
62. Hubbard, S. R. (1997) *EMBO J.* **16,** 5573–5581
63. Duda, T., Goraczniak, R., and Sharma, R. K. (1996) *Biochem. J.* **319,** 279–283
64. Wolbring, G., and Schnetkamp, P. P. M. (1995) *Biochemistry* **34,** 4689–4695
65. Wolbring, G., and Schnetkamp, P. P. M. (1996) *Biochemistry* **35,** 11013–11018
66. Aparicio, J. G., and Applebury, M. L. (1996) *J. Biol. Chem.* **271,** 27083–27089
67. Potter, L. R., and Hunter, T. (1998) *Mol. Cell. Biol.* **18,** 2164–2172
68. Olshevskaya, E. V., and Dizhoor, A. M. (2002) *Association for Research in Vision and Ophthalmology Annual Meeting, Fort Lauderdale, FL, May 5–10, 2002,* Abstract 1413/B423, Association for Research in Vision and Ophthalmology, Bethesda, MD
69. Li, N., Fariss, R. N., Zhang, K., Otto-Bruc, A., Haeseleer, F., Bronson, D., Qin, N., Yamazaki, A., Subbaraya, I., Milam, A. H., Palczewski, K., and Baehr, W. (1998) *Eur. J. Biochem.* **252,** 591–599
70. Bondarenko, V. A., Yu, H., Yamazaki, R. K., and Yamazaki, A. (2002) *Mol. Cell. Biochem.* **230,** 125–128
71. Seno, K., Kishigami, A., Ihara, S., Maeda, T., Bondarenko, V. A., Nishizawa, Y., Usukura, J., Yamazaki, A., and Hayashi, F. (1998) *J. Biol. Chem.* **273,** 22169–22172
72. Barton, G. J. (1993) *Protein Eng.* **6,** 37–40