# Illuminated Rhodopsin Is Required for Strong Activation of Retinal Guanylate Cyclase by Guanylate Cyclase-Activating Proteins†

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ABSTRACT: We have recently shown that activation of retinal guanylate cyclase (retGC) by GC-activating proteins (GCAPs) is much stronger than that previously reported and that preincubation of photoreceptor outer segment homogenates with ATP or its analogue, adenylyl imidodiphosphate (AMP-PNP), is required for the strong activation [Yamazaki, A., Yu, H., Yamazaki, M., Honkawa, H., Matsuura, I., Usukura, J., and Yamazaki, R. K. (2003) *J. Biol. Chem. 278*, 33150−33160]. Here we show that illuminated rhodopsin is essential for development of the AMP-PNP incubation effect. This was demonstrated by illumination of dark homogenates and treatments of illuminated homogenates with 11-*cis*-retinal and hydroxylamine prior to the AMP-PNP incubation and by measurement of the GCAP2 concentration required for 50% activation. We also found that the AMP-PNP incubation effect was not altered by addition of guanosine 5′-*O*-(3-thiotriphosphate), indicating that transducin activation is not required. It is concluded that illuminated rhodopsin is involved in retGC activation in two ways: to initiate the ATP incubation effect for preparation of retGC activation as shown here and to reduce the $Ca^{2+}$ concentrations through cGMP phosphodiesterase activation as already known. These two signal pathways may be activated in a parallel and perhaps proportional manner and finally converge for strong activation of retGC by $Ca^{2+}$-free GCAPs.

Since Bitensky and Miller suggested the involvement of cyclic nucleotide in phototransduction (*1*, *2*), many investigators have contributed to the establishment of the regulation of cGMP metabolism and its consequences for the system. Briefly, after absorption of light, rhodopsin stimulates GTP−GDP exchange on Tα,[1] followed by dissociation of GTP-bound Tα from T$\beta\gamma$. The GTP-bound Tα activates PDE. The resulting decrease in free cGMP concentration leads to closure of cGMP-gated channels and hyperpolarization of plasma membranes. The closure of channels also blocks $Ca^{2+}$ influx, while $Ca^{2+}$ efflux continues through a $Na^+/Ca^{2+}$ exchanger. The resulting decline in the free $Ca^{2+}$ concentration plays a crucial role in light adaptation and recovery processes under dark conditions. Activation of retGCs (also termed ROS-GCs or GC-E and -F) by GCAPs is one of these $Ca^{2+}$-regulated mechanisms.

All membranous GCs, including retGC, are single-transmembrane proteins. The membrane-spanning domain separates an ECD and the intracellular portion composed of a KHD, a dimerization domain, and a catalytic domain (*3*−*5*). In retGC, the ECD is in the intradiscal space and the intracellular domains are in the cytoplasmic space of OS. The identical topography as well as the similarity of intracellular domains (*3*−*5*) suggests a similar regulatory mechanism in membranous GCs. However, in the prevailing model, the mechanism of retGC activation is significantly different from that of peptide-regulated GCs. One of these differences lies in the signal and its reception for their activation. In peptide-regulated GCs, a specific peptide(s) binds to the ECD and the signal is transferred to the intracellular domain for their activation (*3*, *4*, *6*, *7*). In retGC, it has been believed that an outside signal is not required for its activation; i.e., the ECD is not involved in the activation. The immediate signal is the reduction of free $Ca^{2+}$ concentrations in OS (*8*−*10*). $Ca^{2+}$-free GCAPs interact with the intracellular domain of retGC (*11*, *12*) and subsequently stimulate its dimerization (or oligomerization) (*13*, *14*), an essential process for GC activation (*3*, *4*). retGC is not responsive to peptides known to activate GC-A (*5*, *15*, *16*). The negative result is expected because the ECD of retGC shares a low degree of sequence identity with those of peptide-regulated GCs (*5*). A search for peptides to specifically regulate retGC also proved to be futile (*17*).

Another difference between peptide-regulated GCs and retGC lies in the requirement of adenine nucleotide-dependent conformational change for their activation. In the prevailing model for GC-A activation, binding of an atrial

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[1] Abbreviations: Tα and T$\beta\gamma$, subunits of transducin; PDE, cGMP phosphodiesterase 6; retGC, retinal guanylate cyclase; ECD, extracellular domain; KHD, kinase homology domain; GCAPs, guanylate cyclase-activating proteins; OS, outer segments of retinal photoreceptors; AMP-PNP, adenylyl imidodiphosphate; GTP$\gamma$S, guanosine 5′-*O*-(3-thiotriphosphate); DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; $NH_2OH$, hydroxylamine; BSA, bovine serum albumin.

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natriuretic peptide to its ECD facilitates binding of ATP to the KHD (*3*, *4*, *18*, *19*). Once ATP is bound, a conformational change occurs within the KHD that allows the protein to express enzymatic activity. Thus, the ATP-dependent conformational change is essential for its activation. However, the conformational change caused by adenine nucleotide binding is not involved in the current model of retGC activation: its activation requires a simple interaction of retGC with $Ca^{2+}$-free GCAPs (the single-step mechanism). It has been reported that adenine nucleotides affect retGC activity in the assay (*20*−*23*), but the effect has been believed to modify only its activity.

However, we have recently suggested that the adenine nucleotide-dependent conformational change in retGC may be crucial for its activation (*24*). Preincubation of OS homogenates with ATP or AMP-PNP greatly enhances retGC activity stimulated by GCAPs (GCAP1 and -2,) and the strong activation is due to a GCAP-dependent increase in $V_{max}$ without any alteration in the affinity of retGC for GCAPs. Use of AMP-PNP suggests that binding of adenine nucleotides, but not protein phosphorylation, is involved in the incubation effect as an essential step. On the basis of these and other results (*24*), we have proposed a new mechanism for retGC activation (the two-step mechanism). First, ATP binds to retGC (presumably to the KHD) to produce a conformational change in retGC. Then, after reduction of the free $Ca^{2+}$ concentration, retGC is strongly activated by GCAPs. The activation seems to be sufficient to explain the high level of retGC activation estimated in vivo (*25*−*27*). Very recently, we have also shown that this incubation effect is mechanistically different from the adenine nucleotide effect that stimulates retGC activity in the assay (*28*).

Other mechanisms have also been reported to regulate retGC. Illuminated rhodopsin regulates retGC activity (*9*, *25*, *29*−*33*), and the regulation is explained by transducin activation (*25*, *31*−*33*), although the protein targeted by rhodopsin and/or transducin is unknown and the transducin effect is contradictory. We have also shown that the N-terminus of RGS9-1 inhibits retGC activity, basal and GCAP-stimulated (*34*, *35*). These observations imply that retGC regulation may be closely associated with PDE regulation. In addition, phosphorylation of retGC was reported, although its function was unclear (*36*). Stabilization of GCAP-stimulated retGC activity by adenine nucleotides was also reported (*37*); however, its mechanism is unknown. It should be emphasized that these mechanisms have not been integrated into the single-step mechanism for retGC activation.

As described above, our model for retGC activation consists of two steps. As the first step, retGC develops conditions to be activated (the development of the ATP incubation effect), and as the second step, high retGC activity is expressed by GCAPs (the expression of the ATP incubation effect). In this study, we show the role of illuminated rhodopsin in the development of the ATP incubation effect. We treat OS homogenates in three different ways: illumination of dark OS homogenates and treatments of illuminated OS homogenates with 11-*cis*-retinal and $NH_2OH$. Then, we incubate these retGC membranes with AMP-PNP and measure GCAP2-stimulated retGC activity after washing out free AMP-PNP. We find that illuminated rhodopsin is essential for the development, but not the expression, of the AMP-PNP incubation effect. We also show that transducin activation is not involved in signal transduction. On the basis of these results, we propose a new signal transduction mechanism for retGC activation.

## EXPERIMENTAL PROCEDURES

*Materials*. Fresh retinas were isolated from dark-adapted calf eyes obtained from a local slaughterhouse. The dark adaptation was carried out in a light-tight container (3 h, room temperature). Some preliminary studies were also carried out using frozen dark-adapted bovine retinas prepared as described previously (*24*). Dark retinas were also obtained from frogs (*Rana catesbianas* or *Rana grylio*) (*38*). Frogs were dark-adapted overnight and killed without light. 11-*cis*-Retinal was generously supplied by R. K. Crouch (Medical University of South Carolina, Charleston, SC) through the National Eye Institute. GCAP2, a myristoylated form expressed in *Escherichia coli*, was a kind gift from A. M. Dizhoor (Pennsylvania College of Optometry, Elkins Park, PA) and T. Duda and R. K. Sharma (University of Medicine and Dentistry of New Jersey, Piscataway, NJ). A calpain inhibitor, SJA6017, was an obliging gift from J. Inoue (Senju Pharmaceutical Co., Ltd., Kobe, Japan).

*Preparation of OS Homogenates*. Calf OS homogenates were mainly used in this study. OS were isolated from dark-adapted retinas under infrared light (*39*) and suspended in buffer A [10 mM HEPES (pH 7.5), 1 mM DTT, 0.1 mM PMSF, 5 $\mu$M leupeptin, and 5 $\mu$M pepstatin A] supplemented with 20 $\mu$M SJA6017. The suspension (5−10 mg/mL protein) is termed OS homogenates. Preparation of OS from frozen dark-adapted bovine (*24*) and frog (*38*) retinas was carried out as described. After being divided into small portions and frozen with liquid $N_2$, these OS homogenates were stored in the dark at −70 °C. We note that these OS homogenates were prepared without separation of rod and cone OS. Thus, the preparation was a mixture of rod and cone OS, although rod OS are expected to be dominant.

*Preparation of retGC Membranes Incubated with AMP-PNP*. Preparation of these membranes was carried out as described previously (*24*) with slight modification. Briefly, OS homogenates (100 $\mu$g of protein) were suspended with 200 $\mu$L of buffer B [10 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 0.1 mM PMSF, 5 $\mu$M leupeptin, and 5 $\mu$M pepstatin A] and incubated with 5 mM AMP-PNP for 5 min on ice unless otherwise noted. The incubation period was chosen on the basis of the result depicted in Figure 1. The AMP-PNP concentration was chosen on the basis of our previously published data (*24*). After the incubation, samples were centrifuged (350000*g* for 10 min at 4 °C) and membranes were washed with 500 $\mu$L of buffer A supplemented with AMP-PNP (three times) and with 500 $\mu$L of buffer C [10 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 0.1 mM PMSF, 5 $\mu$M leupeptin, 5 $\mu$M pepstatin A, and 100 mM NaCl] (two times). The AMP-PNP concentration in buffer A was same as that of the incubation. The membrane fraction was suspended in buffer B and used as retGC membranes. As a control, OS homogenates were incubated without AMP-PNP and membranes were washed with buffers A and C.

*Pretreatment of OS Homogenates with 11-cis-Retinal or $NH_2OH$*. All procedures for the 11-*cis*-retinal treatment were

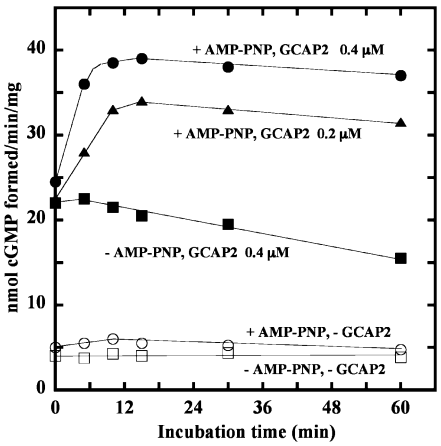


FIGURE 1: Time course of the AMP-PNP incubation effect. Frozen aliquots of OS homogenates were thawed immediately before the AMP-PNP incubation to prevent the loss of GCAP sensitivity before the incubation. The incubation was terminated at the same time by adjusting its starting time. Centrifugation and washing of samples were also carried out at the same time. OS homogenates (100 $\mu$g) were incubated with (○, ●, and ▲) or without (□ and ■) 5 mM AMP-PNP in 200 $\mu$L of buffer B for the indicated periods of time on ice. After the incubation had been terminated by centrifugation, retGC membranes were washed with 500 $\mu$L of buffer C (two times) and suspended in 180 $\mu$L of buffer C. The retGC activity was measured in the presence of various concentrations of GCAP2: 0.4 (● and ■), 0.2 (▲), and 0 $\mu$M (○ and □).

carried out under infrared illumination when isomerization of 11-*cis*-retinal by light was not desirable. First, OS homogenates (150 $\mu$g) were illuminated for 1 s under room light, and then the homogenates were incubated with or without 11-*cis*-retinal in 1 mL of buffer C supplemented with 10% BSA for 2 h on ice. Under these conditions, an ~20-fold molar excess of 11-*cis*-retinal over rhodopsin was present and the final concentration of ethanol, used for dissolving 11-*cis*-retinal, was 0.2% (w/v). After the incubation, excess 11-*cis*-retinal was removed as described previously (*40*) with slight modification. Briefly, 10 $\mu$L of 1 M $NH_2OH$ (pH 7.0) was added to the mixture, and the mixture was further incubated (5 min, on ice). After centrifugation (350000*g* for 15 min at 4 °C), membranes were washed with 1 mL of buffer D [25 mM HEPES (pH 7.5), 1.5 mM $MgCl_2$, 0.5 mM DTT, 5 $\mu$M leupeptin, and 70 mM NaCl] supplemented with 4% (w/v) BSA (two times) and with 1 mL of buffer E (one time). In the case of $NH_2OH$ treatment, illuminated OS homogenates (100 $\mu$g) suspended in 200 $\mu$L of buffer C were incubated with $NH_2OH$ (15−30 min, on ice). Following centrifugation (350000*g* for 10 min at 4 °C), membranes were washed with 500 $\mu$L of buffer C (two times).

*Measurement of retGC Activity*. retGC activity was measured under room or infrared light as described previously (*13*, *15*, *41*) with minor modifications. Briefly, retGC activity was measured with or without GCAP2 in 200 $\mu$L of buffer F [50 mM Tris-HCl (pH 7.5), 2 mM IBMX, 2 mM EGTA, 15 mM phosphocreatine, 10 $\mu$g of creatine phosphokinase, and 10 mM $MgCl_2$]. The GCAP2 concentration was described in each experiment. These concentrations were chosen on the basis of the data previously reported (*24*). When S100B-stimulated retGC activity (*42*) was measured, EDTA was substituted for $CaCl_2$ (100 $\mu$M) and S100B (2 $\mu$M) was added. When retGC activity was measured with $Mn^{2+}$, $MnCl_2$ (2 mM) was added instead of $MgCl_2$. retGC membranes, prepared as described above, were used as the enzyme source (4−7.5 $\mu$g of protein). The reaction (10 min at 33 °C) was started by addition of the mixture of 1 mM (final) GTP ([$\alpha^{32}$P]GTP) and 1 mM (final) cGMP ([$^3$H]-cGMP) and terminated as described. [$^{32}$P]cGMP, the product, and [$^3$H]cGMP were isolated from [$\alpha^{32}$P]GTP as described. The retGC activity in the text is expressed in nanomoles of cGMP formed per minute per milligram (units). Hydrolysis of cGMP, monitored by reduction of [$^3$H]cGMP, was negligible since a $Mg^{2+}$-free hypotonic buffer such as buffer A washed out PDE (data not shown). However, we show the residual concentration of [$^3$H]cGMP after the assay to emphasize that PDE is negligible in these membranes and that retGC activity obtained is not affected by hydrolysis of cGMP even under the conditions in which PDE, if present, could be activated. We also note that retGC-1 was not separated from retGC-2 in all preparations we used. Thus, the retGC activity that was detected is the total activity of retGC-1 and -2, although retGC-1 is expected to be dominant because of the low level of retGC-2 (*43*).

*Other Analytical Methods*. Protein concentrations were measured with BSA as the standard (*44*). SDS−PAGE was performed as described previously (*45*). Proteins in gels were stained with Coomassie blue. In all results, individual points represent the average values of duplicate assays. All experiments were carried out more than three times, and the results were similar. The data shown are representative of these experiments.

## RESULTS

*Determination of the Incubation Period To Show the Effect of Illuminated Rhodopsin.* First, we examined the relationship between the incubation period and the GCAP2-stimulated retGC activity by incubating OS homogenates with AMP-PNP under room light and measuring retGC activity with various concentrations of GCAP2 (Figure 1). We expected that under these conditions OS homogenates would be sufficiently illuminated to fully demonstrate a presumptive light effect on the AMP-PNP incubation. We found that the AMP-PNP incubation enhanced GCAP2-stimulated retGC activity in a time-dependent manner and that the increase reached the maximum within ~7 min when the retGC activity was measured with 0.4 $\mu$M GCAP2 (average of three experiments). The linearity of the increase (up to 10 min) was clearly shown by measuring retGC activity with a lower concentration of GCAP2 (0.2 $\mu$M), indicating that the AMP-PNP effect develops linearly with incubation time (up to 10 min). These results strongly suggest that the presumptive light effect can be measured in a linear range if OS homogenates are incubated for ≤5 min and retGC activity is measured with 0.4 $\mu$M GCAP2. We also found that when the incubation was carried out at higher temperatures, 10 and 20 °C, the retGC activity reached the maximum in less than 5 min (data not shown). These observations indicate that development of the AMP-PNP effect is temperature-dependent, although the effect develops rapidly even on ice. In these experiments, washing of retGC membranes with buffer A supplemented with AMP-PNP was omitted so the AMP-PNP exposure time could be determined precisely. Under these conditions, all activities detected were higher

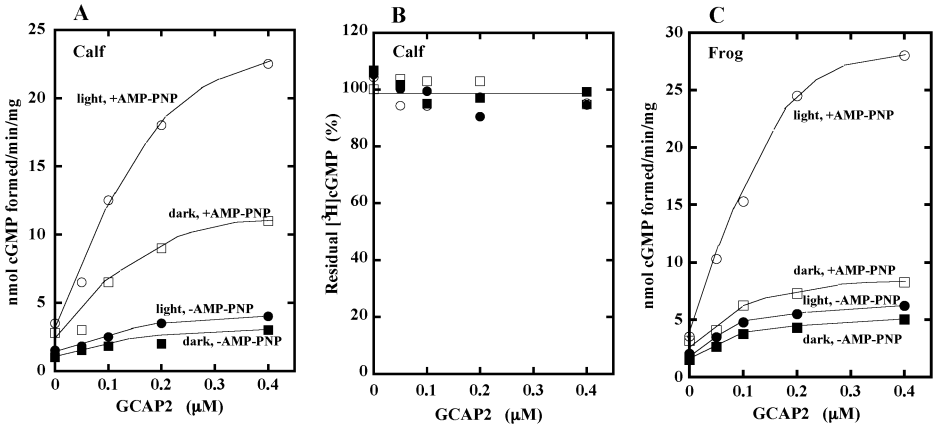


FIGURE 2: Effect of illumination on the AMP-PNP incubation effect. All procedures, except the illumination, were carried out under infrared light. Dark OS homogenates (500 $\mu$g) were divided into four portions. Two portions (□ and ■) were kept in the dark, and the other two portions (○ and ●) were illuminated under room light for 5 min. These OS homogenates (100 $\mu$g) were then incubated (5 min, on ice) with (○ and □) or without (● and ■) 5 mM AMP-PNP in 200 $\mu$L of buffer B. After membranes had been washed, retGC activity was measured with the indicated concentrations of GCAP2. (A) Calf OS homogenates. (B) Residual [$^3$H]cGMP concentrations during and after the assay of retGC activity in panel A. Hydrolysis of cGMP during the assay of retGC activity was monitored using 1 mM [$^3$H]cGMP. (C) Frog OS homogenates.

than those in membranes washed normally because of high basal activity.

When OS homogenates were incubated with AMP-PNP, after the initial increase, only ~5% of retGC activity stimulated by 0.4 $\mu$M GCAP2 decreased in 45 min (from 39.0 units at 15 min to 37.0 units at 60 min). Comparison of retGC activity measured with or without GCAP2 indicates that the slow decline in retGC activity is not GCAP-specific. However, without AMP-PNP, 20−30% of the GCAP2-stimulated activity was reduced in 60 min (from 22.0 to 15.5 units), although the retGC activity barely decreased for the initial ~10 min. The activity measured without GCAP2 appeared to be constant. These results indicate that the ability of retGC to be activated by GCAP2 is unstable in OS homogenates even on ice, but the ability is protected with added AMP-PNP. These results also imply that the AMP-PNP incubation effect, expressed as the increase of GCAP-stimulated retGC activity, declines with time in OS homogenates. This indicates that frozen aliquots of OS homogenates should be thawed immediately before the AMP-PNP incubation and that without AMP-PNP OS homogenates should be incubated for less than 2 h on ice. Tucker et al. (*37*) reported that the ability of retGC to be activated by GCAPs was weakened by incubation at 30−37 °C, but not 4 °C, in washed membranes. The difference between OS homogenates and washed membranes suggests that a washable protein(s) may be involved in the reduction of GCAP-stimulated retGC activity.

*Requirement of Illumination for Strong retGC Activation by the AMP-PNP Incubation.* As described above, retGC is not responsive to peptides known to activate GC-A, and a search for peptides that might regulate retGC proved to be futile. These negative results are understandable, in a sense, because these studies were carried out without adenine nucleotides and/or conditions for expressing adenine nucleotide effects. We extend these studies by investigating whether soluble fractions of OS homogenates were required for the AMP-PNP incubation effect. The result was also negative (data not shown), suggesting that a peptide(s) in OS homogenates, soluble or peripheral, may not be involved in the AMP-PNP effect.

We then examined, using OS homogenates prepared from dark-adapted calf eyes, the possibility that light is a signal for the AMP-PNP incubation effect. We found that illumination of these OS homogenates prior to the AMP-PNP incubation clearly increased GCAP2-stimulated retGC activity (Figure 2A). Without AMP-PNP, the illumination barely increased the GCAP2-stimulated retGC activity. These observations suggest that illumination of OS homogenates is crucial for the AMP-PNP incubation effect. In other words, illuminated rhodopsin is involved in the development and/or expression of the AMP-PNP incubation effect. We note that residual concentrations of [$^3$H]cGMP used to monitor cGMP hydrolysis activity were not changed before and during the assay in all preparations (Figure 2B). This indicates that the cGMP hydrolytic activity is negligible in all preparations and that PDE activity cannot account for the difference between light and dark activities. We also note that illumination similarly increased GCAP1-stimulated retGC activity (data not shown), suggesting that illuminated rhodopsin is also involved in the enhancement of GCAP1-stimulated retGC activity by AMP-PNP preincubation (*24*). We further note that the AMP-PNP incubation effect was always measured under room light in our previous studies (*24*, *28*).

After incubation of these OS homogenates with AMP-PNP, GCAP2-stimulated retGC activities in membrane fractions were measured under infrared and room light. We found that the retGC activity was not altered by light (data not shown). The GCAP2 concentration required for 50% activation (50−70 nM) was also barely changed by illumination (Figure 2A). These observations indicate that the action of illumination on the AMP-PNP incubation effect is exerted before retGC activity is expressed, i.e., illuminated rhodopsin is involved in only the development of the AMP-PNP effect.

It should be noted that the difference between light and dark retGC activities (Figure 2A) was only observed with the AMP-PNP incubation for 5 min. With AMP-PNP incubation for 10 min, both light and dark retGC activities reached a similar maximum and a difference was not evident (data not shown). In addition, GCAP2-stimulated retGC activity in dark membranes incubated with AMP-PNP was

consistently higher than that in membranes incubated without AMP-PNP. These observations suggest that a low-level AMP-PNP incubation effect was developed in calf OS homogenates without illumination. We believe that the effect is due to incomplete dark adaptation. In the calf preparation, all procedures prior to the dark adaptation, killing and harvesting of eyes, were carried out under room light in a local slaughterhouse. Dark adaptation of these eyes was carried out for 3 h at room temperature; however, incomplete dark adaptation is inevitable. Because of this technical difficulty, we were unable to measure the bleaching level to obtain 50% of the AMP-PNP incubation effect or the action spectrum for the effect.

To test our explanation for this technical difficulty, we attempted the same experiment using dark-adapted frog OS homogenates (Figure 2C). Frogs were dark-adapted overnight and killed without light, and OS were isolated under infrared light. We found that only 10−15% of the AMP-PNP incubation effect that developed under saturated light was detected in these dark-adapted frog OS homogenates. In addition, the difference in the AMP-PNP effect in dark and illuminated OS homogenates was observed even after incubation for 10 min (data not shown). These observations strongly suggest that the weak AMP-PNP effect observed in dark-adapted calf OS homogenates (Figure 2A) is, in fact, due to incomplete dark adaptation. We also note that this low level of the AMP-PNP incubation effect was sensitive to $NH_2OH$ (data not shown). As described below, $NH_2OH$ abolishes a rhodopsin isomer(s) required for the development of the AMP-PNP incubation effect. Together, we conclude that the AMP-PNP incubation effect is not developed if OS homogenates are fully dark adapted. We note that frog OS membranes contain two retGCs, retGC-1 and -2, and that the structure of these retGCs is similar to those of bovine retGC (*46*). In addition, their basic characteristics, such as activation by GCAPs, are similar to those of bovine retGC (*46*), although other biochemical characteristics are slightly different from those of bovine retGC (*15*, *46*). Moreover, bovine recombinant GCAPs activate retGC in frog membranes (*46*, *47*). Thus, results obtained by using frog OS homogenates are comparable to those of bovine OS homogenates.

*Reduction of the AMP-PNP Incubation Effect by Addition of 11-cis-Retinal*. We also used 11-*cis*-retinal to determine whether regeneration of rhodopsin weakens the AMP-PNP incubation effect (Figure 3). Illuminated OS homogenates were treated with or without 11-*cis*-retinal, and after excess 11-*cis*-retinal had been washed out, retGC membranes were incubated with or without AMP-PNP. All procedures were carried out under dark conditions (Figure 3, left). With AMP-PNP, GCAP2-stimulated retGC activity in membranes treated with 11-*cis*-retinal (13. 2 units) was clearly lower than that in membranes treated without 11-*cis*-retinal (25.8 units). Without AMP-PNP, the retGC activities were barely changed in membranes treated with or without 11-*cis*-retinal. These observations suggest that the AMP-PNP incubation effect, its development, and/or expression are sensitive to the 11-*cis*-retinal treatment.

It should be emphasized that the treatment with 11-*cis*-retinal did not reduce GCAP2-stimulated retGC activity to the level in membranes incubated without AMP-PNP (Figure 3, left). We believe that this is due to the following technical

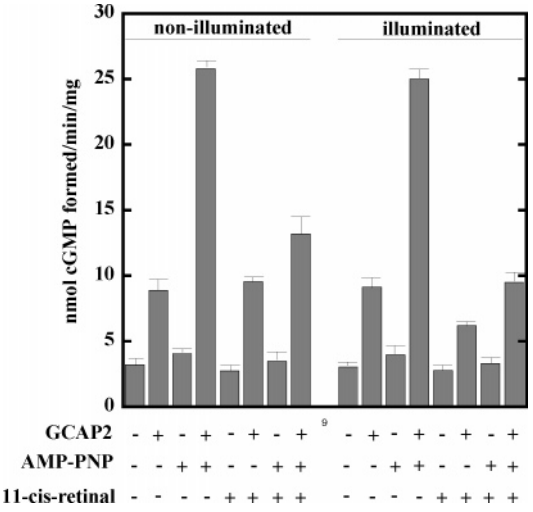


FIGURE 3: Effect of 11-*cis*-retinal on the AMP-PNP incubation effect. Dark OS homogenates were illuminated for 1 s under room light. For the nonilluminated samples, after the initial illumination, all procedures were carried out under infrared light. For the illuminated samples, after the initial illumination, the second illumination was performed prior to the AMP-PNP incubation. All subsequent experiments were carried out in the dark. In each sample, the homogenate (150 $\mu$g) was incubated with or without a 20-fold molar excess of 11-*cis*-retinal in 1 mL of buffer E for 2 h on ice. After excess 11-*cis*-retinal had been removed, retGC membranes were incubated with or without 5 mM AMP-PNP in 1 mL of buffer A (5 min, on ice). After membranes had been washed, retGC activity in membranes was measured with or without 0.5 $\mu$M GCAP2.

difficulty. In general, a long incubation (12−24 h, on ice) is required for complete regeneration of rhodopsin form opsin with 11-*cis*-retinal (*40*, *48*). However, such a long incubation could not be carried out, since, without AMP-PNP, the ability of retGC to be activated by GCAPs in OS homogenates slowly but consistently declines during incubation even on ice (Figure 1). We incubated OS homogenates with 11-*cis*-retinal for 2 h on ice. Thus, rhodopsin might not be completely regenerated, and its active forms might be still present and develop the small AMP-PNP incubation effect. We note that the concentration of added 11-*cis*-retinal was higher than usual (*48*) to accelerate the regeneration of rhodopsin. Thus, measurement of the concentration of regenerated rhodopsin was difficult because of the high 11-*cis*-retinal concentration (*48*).

We also examine the effect of illumination on retGC activity in membranes pretreated with 11-*cis*-retinal (Figure 3, right). The illumination was performed after incubation with 11-*cis*-retinal and washing out of 11-*cis*-retinal but prior to AMP-PNP incubation, and after the illumination, all processes were carried out under dark conditions. Surprisingly, the illumination further reduced GCAP-stimulated retGC activity in membranes treated with 11-*cis*-retinal. With AMP-PNP, the activity was reduced from 13.2 to 9.50 units (27.7%). Without AMP-PNP, the activity was also decreased from 9.55 to 6.20 units (35.1%). However, without GCAP2, the illumination barely reduced retGC activity. These observations suggest that a factor produced by illumination inhibits the interaction of retGC with GCAPs. The detailed mechanism for the inhibition is unknown. However, it is possible that significant amounts of residual 11-*cis*-retinal might be still bound to a protein(s) other than opsin and/or

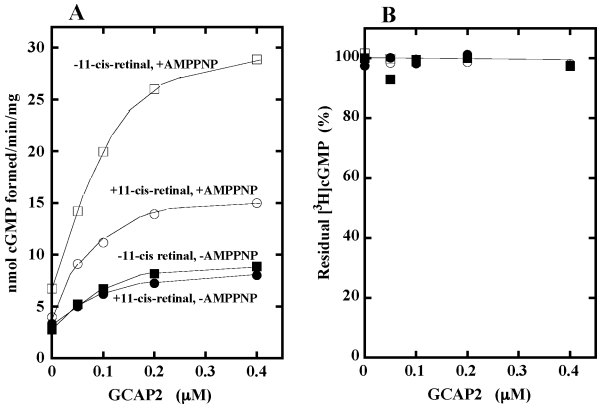


FIGURE 4: Effect of 11-*cis*-retinal on the AMP-PNP incubation effect. retGC activity measured with various concentrations of GCAP2. (A) Effect of 11-*cis*-retinal. Dark OS homogenates were illuminated for 1 s under room light. After the illumination, all procedures were carried out under infrared light. The homogenate (150 $\mu$g) was incubated with (○ and ●) or without (□ and ■) a 20-fold molar excess of 11-*cis*-retinal in 1 mL of buffer E for 2 h on ice. After excess 11-*cis*-retinal had been removed, retGC membranes were incubated with (○ and □) or without (● and ■) 5 mM AMP-PNP in 1 mL of buffer A (5 min, on ice). After membranes had been washed, retGC activity in membranes was measured with the indicated concentrations of GCAP2. (B) Residual [$^3$H]cGMP concentration during the assay of retGC activity. Hydrolysis of cGMP during the assay of retGC activity in panel A was monitored using 1 mM [$^3$H]cGMP. Symbols are the same as those in panel A.

to membranes, although we washed out excess 11-*cis*-retinal, and that after illumination a photoisomer(s) of 11-*cis*-retinal, possibly all-*trans*-retinal, might weaken the interaction of retGC with GCAP2. The possible binding of all-*trans*-retinal to membranes and its inhibitory effect on cGMP-gated channels have been described previously (*49*).

The result described above also implies the possibility that the reduction of GCAP2-stimulated retGC activity by 11-*cis*-retinal (Figure 3 left) is due to inhibition of the interaction of retGC with GCAP2 by 11-*cis*-retinal bound to a site(s) other than opsin. To rule out this possibility, we examined the effect of 11-*cis*-retinal on the AMP-PNP incubation effect by changing GCAP2 concentrations in the assay (Figure 4). All procedures were carried out under dark conditions. We found again that 11-*cis*-retinal specifically reduces GCAP2-stimulated retGC activity in membranes treated with AMP-PNP (Figure 4A). Under the conditions, the GCAP2 concentration required for 50% activation was barely changed with or without 11-*cis*-retinal (50−70 nM). This implies that the interaction of retGC with GCAP2 is not affected by 11-*cis*-retinal regardless of its binding site. In other words, exogenous 11-*cis*-retinal inhibits the development of the AMP-PNP incubation effect.

We note that residual contents of [$^3$H]cGMP used to monitor cGMP hydrolysis activity were not changed before and during the assay (Figure 4B), indicating that cGMP hydrolytic activity is negligible in all membrane preparations before and during the assay. We also note that BSA was added to the incubation with 11-*cis*-retinal and a washing buffer to remove excess 11-*cis*-retinal. However, BSA changed neither the AMP-PNP incubation effect (up to 10% BSA) nor the GCAP2-stimulated retGC activity (up to 1% BSA). Thus, all results described above are consistent with our assumption that 11-*cis*-retinal slows the development of the AMP-PNP incubation effect. However, we cannot rule out the possibility that 11-*cis*-retinal bound to a site(s) other than opsin also suppresses the development in an unknown manner.

*Reduction of the AMP-PNP Incubation Effect by Hydroxylamine Pretreatment.* As described above, the experiment using 11-*cis*-retinal presents technical difficulties, and the conclusion that is obtained is circumstantial. Thus, we also used another method, pretreatment of illuminated OS homogenates with $NH_2OH$, to show the involvement of illuminated rhodopsin in the AMP-PNP incubation effect. It is known that $NH_2OH$ treatment of bleached OS membranes causes a rapid decay of the active species of rhodopsin required for transducin activation (*50*). First, as controls, we examined the $NH_2OH$ effect in membranes incubated without AMP-PNP. After pretreatment with $NH_2OH$ and washing out of excess $NH_2OH$, OS membranes were incubated without AMP-PNP, and the retGC activity in membranes was measured with $Mn^{2+}$, GCAP2, or S100B (Figure 5A). We found that retGC activity was slightly but consistently increased by the treatment under all the assay conditions that were tested and that a linear relationship existed between the increase and the $NH_2OH$ concentrations. Residual concentrations of [$^3$H]cGMP used to monitor cGMP hydrolysis activity were barely changed (Figure 5B), indicating that the cGMP hydrolytic activity was negligible and the PDE activity was not altered by either the pretreatment with $NH_2OH$ or components added to the assay. These results give a clear indication that the catalytic domain of retGC was not adversely affected by the $NH_2OH$ treatment. These observations also suggest that the $NH_2OH$ treatment increases retGC activity by affecting a characteristic(s) of retGC common to all the assay conditions that were tested.

However, the $NH_2OH$ pretreatment differently affected GCAP2-stimulated retGC activity in membranes incubated with AMP-PNP (Figure 5C). The retGC activity declined with the increase in the $NH_2OH$ concentration (up to 100 mM) and then increased. Extrapolation of the GCAP2-stimulated retGC activity in membranes incubated without AMP-PNP (Figure 5A) suggests that $NH_2OH$ (100 mM) reduces the retGC activity to the level detected in membranes incubated without AMP-PNP; i.e., $NH_2OH$ (100 mM) abolishes ∼100% of the AMP-PNP incubation effect. The extrapolation also suggests that the increase in the retGC activity above 100 mM $NH_2OH$ is similar to that in membranes incubated without AMP-PNP; i.e., after the AMP-PNP incubation effect had been completely abolished, only the nonspecific activation of retGC by $NH_2OH$ (Figure 5A) was observed. We note that the treatment with $NH_2OH$ (100 mM) completely inhibited GTP$\gamma$S-dependent PDE activity in OS homogenates (data not shown), indicating that illuminated rhodopsin is abolished by the treatment. Residual concentrations of [$^3$H]cGMP used to monitor cGMP hydrolysis activity were barely changed (Figure 5D), indicating that the cGMP hydrolytic activity is negligible and residual PDE was activated by neither the $NH_2OH$ treatment nor assay components. Together, these observations strongly suggest that illuminated rhodopsin is required for the AMP-PNP effect, its development, and/or expression.

We also checked the effect of $NH_2OH$ pretreatment on retGC activity measured with different GCAP2 concentrations (Figure 6). OS homogenates were incubated with or

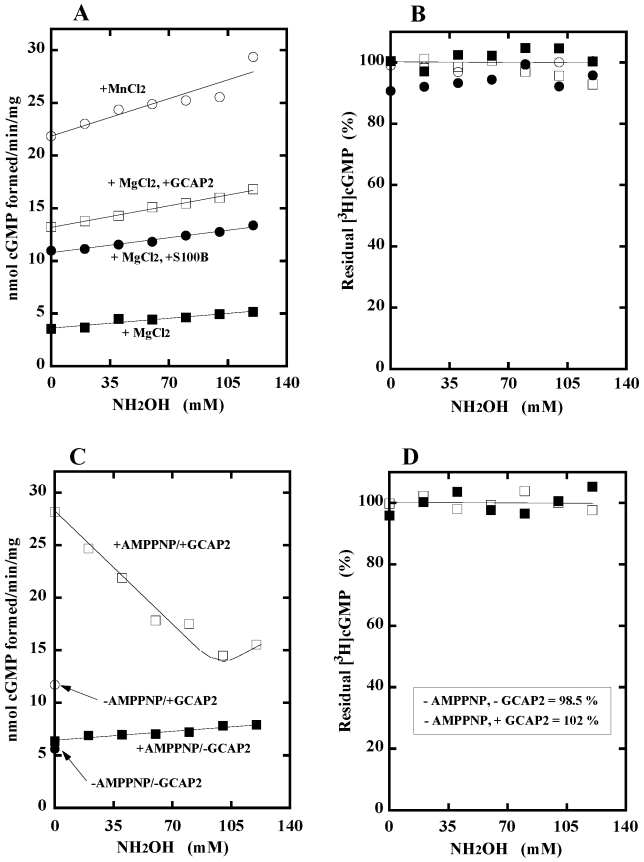


FIGURE 5: Effect of $NH_2OH$ pretreatment on the AMP-PNP incubation effect. OS homogenates (100 $\mu$g) in 200 $\mu$L of buffer C were incubated with the indicated concentrations of $NH_2OH$ (20 min, on ice). After excess $NH_2OH$ had been washed out with 500 $\mu$L of buffer C (twice), membranes were incubated with or without 5 mM AMP-PNP in 200 $\mu$L of buffer A (5 min, on ice). (A) retGC activity in membranes incubated without AMP-PNP. retGC activity was measured with various components: (○) $MnCl_2$ (2 mM), (□) $MgCl_2$ (10 mM) and GCAP2 (0.5 $\mu$M), (●) $MgCl_2$ (10 mM) and S100B (2 $\mu$M), and (■) $MgCl_2$ (10 mM). (B) Residual [$^3$H]cGMP concentrations after the assay of retGC activity in panel A. Hydrolysis of cGMP during the assay of retGC activity was monitored using 1 mM [$^3$H]cGMP. Symbols are the same as those in panel A. (C) retGC activity in membranes incubated with AMP-PNP. retGC activity was measured with (□) or without (■) GCAP2 (0.5 $\mu$M). As controls, retGC activity in membranes incubated without AMP-PNP was also measured with (○) or without (●) GCAP2 (0.5 $\mu$M). (D) Residual [$^3$H]cGMP concentrations after the retGC activity assay in panel C. Symbols are the same as those in panel C.

without 50 mM $NH_2OH$, and excess $NH_2OH$ was washed out prior to the incubation with or without AMP-PNP (Figure 6A). With AMP-PNP, the $NH_2OH$ pretreatment minimally changed the basal activity (the activity measured without GCAP2); however, the treatment reduced retGC activity measured with GCAP2. Without AMP-PNP, the treatment did not change all retGC activities. Again, residual concentrations of [$^3$H]cGMP used to monitor cGMP hydrolysis activity were not changed (Figure 6B). The GCAP2 concentrations required for the 50% stimulation were barely changed with or without $NH_2OH$ (50−65 nM), indicating that the $NH_2OH$ treatment does not change the interaction between retGC and GCAP2. This implies that expression of the AMP-PNP effect is not altered by the $NH_2OH$ treatment; i.e., only the development of the AMP-PNP effect is sensitive to the $NH_2OH$ treatment. Together with results depicted in

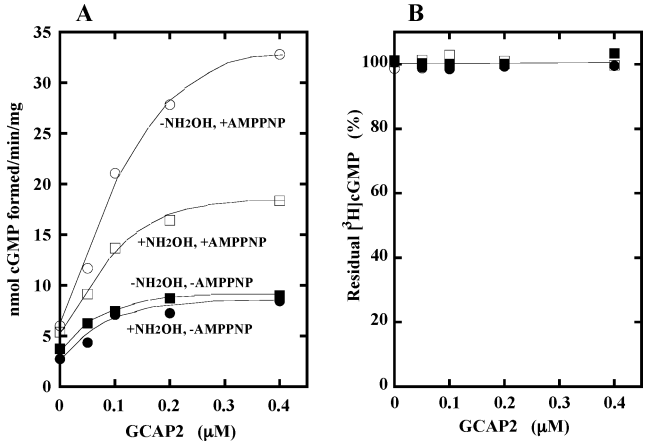


FIGURE 6: Effect of $NH_2OH$ pretreatment on the AMP-PNP incubation effect. retGC activity measured with various concentrations of GCAP2. OS homogenates (100 $\mu$g) were treated with (● and □) or without (○ and ■) 50 mM $NH_2OH$ in 600 $\mu$L of buffer C (20 min, on ice). After excess $NH_2OH$ had been washed out, membranes were incubated with (○ and □) or without (● and ■) 5 mM AMP-PNP. After membranes had been washed, retGC activity was measured with the indicated concentrations of GCAP2. (A) GCAP2-stimulated retGC activity. (B) Residual [$^3$H]cGMP concentrations after the retGC activity assay. Hydrolysis of cGMP during the assay of retGC activity in panel A was monitored using 1 mM [$^3$H]cGMP. Symbols are the same as those in panel A.

Figures 2−4, our observations strongly suggest that an active species of rhodopsin is required for the development of the AMP-PNP incubation effect.

*No Involvement of Transducin in the AMP-PNP Incubation Effect*. Rhodopsin is the prototypical receptor for G protein-mediated signal transduction. Thus, the effect of bleached rhodopsin might be mediated by transducin. However, in our previous studies (*24*, *28*), the AMP-PNP incubation effect was observed without adding GTP or its analogues to the AMP-PNP incubation. This strongly suggests that transducin is not involved in the signal transfer from illuminated rhodopsin to the AMP-PNP incubation effect. To establish this further, we examined the effect of added GTP$\gamma$S on the AMP-PNP incubation effect (Figure 7A). The incubation was carried out under room light. We found that GTP$\gamma$S (up to 100 $\mu$M) did not alter the GCAP2-stimulated retGC activity enhanced by AMP-PNP incubation. Again, residual concentrations of [$^3$H]cGMP used to monitor cGMP hydrolysis activity were not changed (Figure 7B), indicating that the PDE content in membranes was negligible and residual PDE was not newly activated by components in the assay.

We also tried to confirm these observations by measuring retGC activity with various concentrations of GCAP2 (Figure 7C). We found that GTP$\gamma$S (100 $\mu$M) did not affect GCAP2-stimulated retGC activity in membranes incubated with AMP-PNP. The GCAP2 concentrations required for the 50% stimulation (40−65 nM) were minimally changed with or without GTP$\gamma$S, indicating that addition of GTP$\gamma$S does not alter the interaction of retGC with GCAP2. Again, residual concentrations of [$^3$H]cGMP used to monitor cGMP hydrolysis activity were not changed (Figure 7D). We also examined the possibility that the transducin in the OS homogenates that were used might not be functional. We washed membranes with buffer C and examined the solubilization of T$\alpha$ (39 kDa) and T$\beta$ (36 kDa) by GTP$\gamma$S from

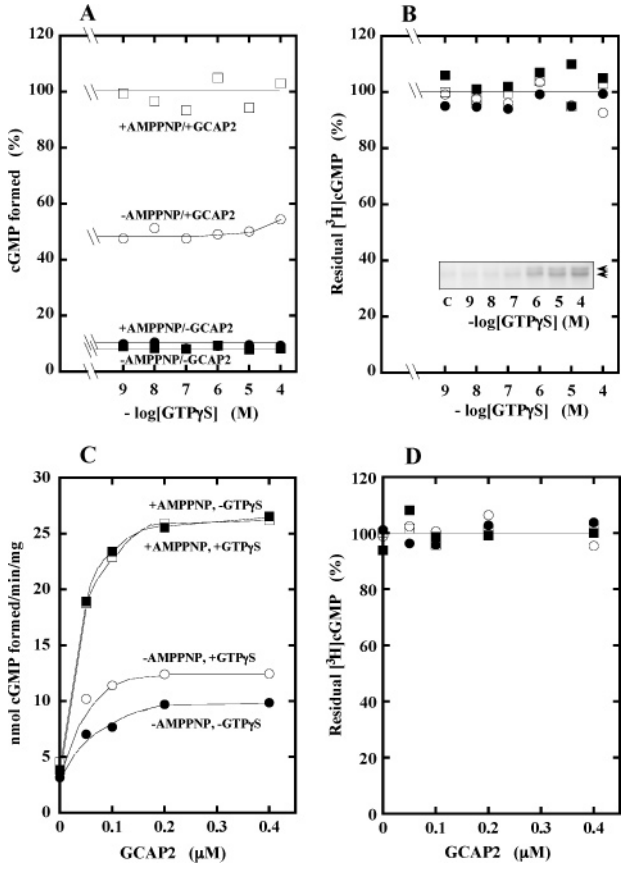


FIGURE 7: Effect of GTP$\gamma$S on the AMP-PNP incubation effect. (A) GTP$\gamma$S concentrations. OS homogenates (100 $\mu$g) suspended in 200 $\mu$L of buffer B were incubated with (□ and ■) or without (○ and ●) 5 mM AMP-PNP in the presence of the indicated concentrations of GTP$\gamma$S. After free nucleotides had been washed out with 500 $\mu$L of buffer A with (□ and ■) or without (○ and ●) 5 mM AMP-PNP (three times) and 500 $\mu$L of buffer C (two times), membranes were suspended in 120 $\mu$L of buffer C and retGC activity in membranes was measured with (□ and ○) or without (■ and ●) 0.4 $\mu$M GCAP2. The 100% activity was 28.8 nmol of cGMP formed $min^{-1}$ $mg^{-1}$. (B) Residual [$^3$H]cGMP concentrations after the retGC activity assay. Hydrolysis of cGMP during the assay of retGC activity in panel A was monitored using 1 mM [$^3$H]cGMP. Symbols are the same as those in panel A. The inset in panel B shows SDS−PAGE of soluble fractions. After membranes had been washed in OS homogenates with buffer C, T$\alpha$ (39 kDa) and T$\beta$ (36 kDa) were solubilized with the indicated concentrations of GTP$\gamma$S and detected by SDS−PAGE. Lane C was without GTP$\gamma$S. (C) retGC activity measured with different GCAP2 concentrations. OS homogenates (100 $\mu$g) suspended in 200 $\mu$L of buffer B were incubated with (□ and ■) or without (○ and ●) 5 mM AMP-PNP in the presence (□ and ○) or absence (■ and ●) of 100 $\mu$M GTP$\gamma$S for 15 min on ice. After centrifugation, membranes were washed as described in panel A. retGC activity was measured with the indicated concentrations of GCAP2. (D) Residual [$^3$H]cGMP concentrations after the retGC activity assay. Symbols are the same as those in panel C.

the membranes. We found that added GTP$\gamma$S (>0.1 $\mu$M) solubilized both transducin subunits (Figure 7B, inset), indicating that transducin was functional. Together, our observations indicate that transducin activation is not required for the AMP-PNP incubation effect.

GCAP2-stimulated retGC activity was slightly increased when OS homogenates were incubated with GTP$\gamma$S (>0.1 $\mu$M) (Figure 7A). The slight increase was also observed in retGC activity measured with various concentrations of GCAP2 (Figure 7C). However, the increase was not evident when AMP-PNP was added to the incubation (Figure 7A,C). Under these conditions, PDE and its activity were negligible in these membranes, as described above, and transducin subunits were also washed out. Thus, a change of characteristics in an OS protein(s), other than PDE, by GTP$\gamma$S-bound T$\alpha$ or T$\beta\gamma$ and/or loss of transducin subunits may enhance the GCAP2-stimulated retGC activity. We note that the concentration of GTP$\gamma$S required for the increase is greater than 0.1 $\mu$M (Figure 7A), suggesting that endogenous GTP in OS homogenates is negligible. This implies that the absence of an effect of GTP$\gamma$S on the AMP-PNP incubation effect (Figure 7A,C) is not due to the presence of saturated amounts of endogenous GTP in OS homogenates.

## DISCUSSION

We have shown that preincubation of OS homogenates with ATP or AMP-PNP greatly enhances GCAP-stimulated retGC activity (*24*) and that the incubation effect is mechanistically different from an adenine nucleotide effect that stimulates retGC activity in the assay (*28*). In this study, we have suggested the illuminated rhodopsin, a typical receptor for G protein-mediated signal transduction, is essential for development, but not expression, of the AMP-PNP incubation effect; however, transducin, the retinal G protein, is not involved in signal transduction. On the basis of these results and our knowledge of PDE activation, we propose that illumination of rhodopsin is involved in retGC activation in two ways. It generates the ATP incubation effect on retGC, as shown here, and it reduces the $Ca^{2+}$ concentration through PDE activation, as already established. These two pathways may be activated in a parallel and perhaps proportional manner and then converge to produce the large retGC activation by $Ca^{2+}$-free GCAPs.

We used three different methods to show the requirement of illuminated rhodopsin for the AMP-PNP incubation effect: illumination of dark OS membranes (Figure 2) and pretreatment of illuminated OS homogenates with 11-*cis*-retinal (Figures 3 and 4) and $NH_2OH$ (Figures 5 and 6). The first method had an inevitable technical difficulty in preparation of dark-adapted calf OS homogenates; however, the difficulty was overcome by using dark-adapted frog OS homogenates. The second method also had two technical difficulties, incomplete regeneration of rhodopsin under the experimental conditions and an inability to eliminate the effect of 11-*cis*-retinal bound to a site(s) other than opsin. We could not overcome these difficulties. Thus, the conclusions are based on indirect evidence. However, results are consistent with our assumption that illumination is required for the development of the AMP-PNP effect. In experiments using the third method, we clearly showed that pretreatment of OS homogenates with $NH_2OH$ completely and specifically inhibited the development of the AMP-PNP incubation effect. Together, we conclude that illuminated rhodopsin is essential for development of the AMP-PNP incubation effect. On the basis of the results described here and previously (*24*), we propose now that the light signal accepted by rhodopsin is transduced into the binding of ATP to retGC (provably to the KHD) and that ATP binding changes its conformation for the subsequent activation.

Previous studies also suggested that illuminated rhodopsin is involved in retGC regulation (*9*, *25*, *29*−*33*). In electro-

physiological studies, Kondo and Miller (*31*) reported that light accelerated retGC activity. Cornwall and Fain (*25*) also reported that the retGC rate was accelerated by illumination and that the estimated steady-state retGC velocity appeared to be a linear function of the amount of pigment bleached. Further, the illumination effect was reversed only if the photopigment was regenerated. These results are consistent with our conclusion, indicating that the mechanism we have proposed is physiologically relevant to retGC activation. They interpreted these observations as being the result of transducin activation; however, such an interpretation is understandable since illuminated rhodopsin has a major role in activating PDE through transducin activation in phototransduction.

Biochemical studies also reported the involvement of rhodopsin and/or transducin in retGC regulation. Goldberg and co-workers (*29*) have reported a light-accelerated rate of cGMP synthesis; however, they did not describe the $Ca^{2+}$ sensitivity of retGC activity. Pepe and co-workers have reported that illumination increases $Ca^{2+}$-sensitive retGC activity (*9*, *30*) and that an antibody specific to Tα suppresses the increase (*32*). They did not use ATP or its analogue in any stage of their experiments. Thus, their results may not be related to our results. On the other hand, Schnetkamp and co-workers (*33*) have reported that light inhibits GCAP1-dependent retGC activity and that the inhibition is dependent on T$\alpha\beta\gamma$ or T$\beta\gamma$. Their finding may be related to our result which shows that GCAP2-stimulated retGC activity was slightly increased when OS homogenates were incubated with only GTP$\gamma$S and washed (Figure 7), since loss of transducin subunits may be a reason for the increase. However, we did not further investigate the mechanism because the stimulation we observed was weak. In addition, we could not envision the stimulation in vivo since the stimulation is abolished by AMP-PNP (Figure 7), but the ATP concentration in rod OS may not be reduced. Previous studies reported that the ATP concentration in rod OS is 3−4 mM (*51*) and not reduced by light (*52*).

In this study, in contrast to the studies cited above, we suggest that transducin is not involved in signal transduction from illuminated rhodopsin to retGC. It should be noted that other groups have already reported that some illumination effects are not dependent on transducin subunits. Balasubramanian and Slepak (*53*) reported that the small G protein Rac-1 is directly activated by illuminated rhodopsin. Also, studies of light-dependent arrestin translocation indicated that this process does not require active transducin subunits (*54*, *55*). In addition, Woodruff et al. (*56*) reported transducin-independent light-activated $Ca^{2+}$ release. If so, how is the light signal transferred to retGC? Two ways, direct and indirect, may be possible. In the direct way, rhodopsin directly interacts with retGC and the interaction may occur in cytoplasmic or intradiscal spaces. In the cytoplasmic space, a cytoplasmic loop(s) of rhodopsin may interact with the intracellular domain of retGC and the ECD would not be involved. In the intradiscal space, the ECD would be involved. The E-II loop of rhodopsin (*57*) is a candidate for interacting with the ECD because its conformation is changed by light (*58*−*63*). In the indirect way, rhodopsin would interact with retGC through a mediator (other than transducin) and the interaction may also occur in cytoplasmic or intradiscal spaces. In the cytoplasmic space, a cytoplasmic loop(s) of rhodopsin may interact with the intracellular domain of retGC through a mediator and the ECD would not be involved. In the interdiscal space, a mediator might interact with rhodopsin and the ECD. The E-II loop of rhodopsin is also a candidate for interacting with a mediator. An extract in $Mg^{2+}$-free hypotonic buffers (*28*) may allow identification of a putative new mediator.

Here we have shown that illumination is essential for the development of the AMP-PNP incubation effect. This leads to our model in which retGC is activated by GCAPs only when retGC changes its conformation upon binding of ATP to the KHD. However, it is also true that retGC is stimulated by GCAPs without ATP binding, although the level of stimulation is low. How can we explain this low activity in our model? As discussed (*24*), we presume two retGC conformations in equilibrium: an inactive form without enzyme activity and possessing no ability to be stimulated by GCAPs and an active form with enzyme activity that is dependent on GCAPs. Without ATP binding, only a small fraction of retGC exists in the active form. The small increase in the retGC activity by exogenous GCAP2 would be due to the portion of the active form whose GCAP2 sites are not filled by endogenous GCAP2. Thus, exogenous GCAP2 binds to the residual GCAP-free active form to express retGC activity; however, the observed retGC activity is not large because the fraction of retGC present as the active form is small. Treatment with ATP (or AMP-PNP) in the presence of illuminated rhodopsin produces ATP binding that shifts the equilibrium from the inactive form toward the active form, and a large retGC activity is expressed if enough GCAP is present. This concept is clearly supported by our findings that the strong activation is obtained without changing the affinity of retGC for GCAPs and the level of activation is increased with AMP-PNP concentrations (*24*).

In summary, we propose that illumination of rhodopsin is a trigger for two signal pathways for retGC activation and that these two pathways ultimately converge for strong activation of retGC by $Ca^{2+}$-free GCAPs. Obviously, further studies are needed. Mechanisms for the interaction of retGC with illuminated rhodopsin followed by binding of ATP to retGC and for the turnoff of the AMP-PNP incubation effect especially should be explored to establish our model. However, it is clear now that our model for retGC activation fits the function of retGC, rapid replenishment of cGMP in OS after its reduction by PDE.

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## REFERENCES

1. Bitensky, M. W., Gorman, R. E., and Miller, W. H. (1971) Adenyl cyclase as a link between photon capture and changes in membrane permeability of frog photoreceptors, *Proc. Natl. Acad. Sci. U.S.A. 68*, 561−562.
2. Miller, W. H. (1990) Dark Mimic, *Invest. Ophthalmol. Visual Sci. 31*, 1664−1673.
3. Lucas, K. A., Pitari, G. M., Kazerounian, S., Ruiz-Stewart, I., Park, J., Schulz, S., Chepenik, K. P., and Waldman, S. A. (2000) Guanylyl cyclases and signaling by cGMP, *Pharmacol. Rev. 52*, 375−413.

4. Potter, L. R., and Hunter, T. (2001) Guanylyl cyclase-linked natriuretic peptide receptors: Structure and regulation, *J. Biol. Chem. 276*, 6057−6060.
5. Goraczniak, R. M., Duda, T., Sitaramayya, A., and Sharma, R. K. (1994) Structure and functional characterization of the rod outer segment membranes guanylate cyclase, *Biochem. J. 302*, 455−461.
6. van den Akker, F. (2001) Structural insights into the ligand binding domains of membrane bound guanylyl cyclases and natriuretic peptide receptors, *J. Mol. Biol. 311*, 923−937.
7. Misono, K. S. (2002) Natriuretic peptide receptor: Stricture and signaling, *Mol. Cell. Biochem. 230*, 46−90.
8. Lolley, R. N., and Racz, E. (1982) Calcium modulation of cyclic GMP synthesis in rat visual cells, *Vision Res. 22*, 1481−1486.
9. Pepe, I. M., Panfoli, I., and Cugnoli, C. (1986) Guanylate cyclase in rod outer segments of the toad retina: Effect of light and $Ca^{2+}$, *FEBS Lett. 203*, 73−76.
10. Koch, K.-W., and Stryer, L. (1988) Highly cooperative feedback control of retinal rod guanylate cyclase by calcium ions, *Nature 334*, 64−66.
11. Laura, R. P., Dizhoor, A. M., and Hurley, J. B. (1996) The membrane guanylyl cyclase, retinal guanylyl cyclase-1, is activated through its intracellular domain, *J. Biol. Chem. 271*, 11646−11651.
12. Duda, T., Goraczniak, R., Surgucheva, I., Rudnicka-Nawrot, M., Gorczyca, W. A., Palczewski, K., Sitaramayya, A., Baehr, W., and Sharma, R. K. (1996) Calcium modulation of bovine photoreceptor guanylate cyclase, *Biochemistry 35*, 8478−8482.
13. Yu, H., Olshevskaya, E., Duda, T., Seno, K., Hayashi, F., Sharma, R. K., Dizhoor, A. M., and Yamazaki, A. (1999) Activation of retinal guanylyl cyclase-1 by $Ca^{2+}$-binding proteins involves its dimerization, *J. Biol. Chem. 274*, 15547−15555.
14. Ramamurthy, V., Tucker, C., Wilkie, S. E., Daggett, V., Hunt, D. M., and Hurley, J. B. (2001) Interactions within the coiled-coil domain of retGC-1 guanylyl cyclase are optimized for regulation rather than for high affinity, *J. Biol. Chem. 276*, 26218−26229.
15. Hayashi, F., and Yamazaki, A. (1991) Polymorphism in purified guanylate cyclase from vertebrate rod photoreceptors, *Proc. Natl. Acad. Sci. U.S.A. 88*, 4746−4750.
16. Yang, R.-B., Foster, D. C., Garbers, D. L., and Fulle, H.-J. (1991) Two membrane forms of guanylyl cyclase found in the eye, *Proc. Natl. Acad. Sci. U.S.A. 92*, 602−606.
17. Dizhoor, A. M., and Hurley, J. B. (1999) Regulation of photoreceptor membrane guanylyl cyclases by guanylyl cyclase activating proteins, *Methods 19*, 521−531.
18. Sharma, R. K., Yadav, P., and Duda, T. (2002) Allosteric regulatory step and configuration of the ATP-binding pocket in atrial natriuretic factor receptor guanylate cyclase transduction mechanism, *Can. J. Physiol. Pharmacol. 79*, 682−691.
19. Joubert, S., Jossart, C., McNicoll, N., and De Lean, A. (2005) Atrial natriuretic peptide-dependent photolabeling of a regulatory ATP-binding site on the natriuretic peptide receptor-A, *FASEB J. 272*, 5572−5583.
20. Krishnan, N., Fletcher, R. T., Chader, G. J., and Krishna, G. (1978) Characterization of guanylate cyclase of rod outer segments of the bovine retina, *Biochim. Biophys. Acta 523*, 506−515.
21. Sitaramayya, A., Marala, R. B., Hakki, S., and Sharma, R. K. (1991) Interactions of nucleotide analogues with rod outer segment guanylate cyclase, *Biochemistry 30*, 6742−6747.
22. Gorczyca, W. A., van Hooser, P., and Palczewski, K. (1994) Nucleotide inhibitors and activators of retinal guanylyl cyclase, *Biochemistry 33*, 3217−3222.
23. Sitaramayya, A., Duda, T., and Sharma, R. K. (1995) Regulation of bovine rod outer segment membrane guanylate cyclase by ATP, phosphodiesterase and metal ions, *Mol. Cell. Biochem. 148*, 139−145.
24. Yamazaki, A., Yu, H., Yamazaki, M., Honkawa, H., Matsuura, I., Usukura, J., and Yamazaki, R. K. (2003) A critical role for ATP in the stimulation of retinal guanylyl cyclase by guanylyl cyclase-activating proteins, *J. Biol. Chem. 278*, 33150−33160.
25. Cornwall, M. C., and Fain, G. L. (1994) Bleached pigment activates transduction in isolated rods of the salamander retina, *J. Physiol. 480*, 261−279.
26. Koutalos, Y., Nakatani, K., Tamura, T., and Yau, K.-W. (1995) Characterization of guanylate cyclase activity in single retinal rod outer segments, *J. Gen. Physiol. 106*, 863−890.
27. Detwiler, P. D. (2002) Open the loop: Dissecting feedback regulation of a second messenger transduction cascade, *Neuron 36*, 3−4.
28. Yamazaki, M., Usukura, J., Yamazaki, R. K., and Yamazaki, A. (2005) ATP binding is required for physiological activation of retinal guanylate cyclase, *Biochem. Biophys. Res. Commun. 338*, 1291−1298.
29. Ames, A., III, Walseth, T. F., Heyman, R. A., Barad, M., Graeff, R. M., and Goldberg, N. D. (1986) Light-induced increase in cGMP metabolic flux correspond with electrical responses of photoreceptors, *J. Biol. Chem. 261*, 13034−13042.
30. Pepe, I. M., Boero, A., Vergani, L., Panfoil, I., and Cognoli, C. (1986) Effect of light and calcium on cyclic GMP synthesis in rod outer segments of toad retina, *Biochim. Biophys. Acta 889*, 271−276.
31. Kondo, H., and Miller, W. H. (1988) Rod light adaptation may be mediated by acceleration of the phosphodiesterase-guanylate cyclase cycle. *Proc. Natl. Acad. Sci. U.S.A. 85*, 1322−1326.
32. Pepe, I. M., Panfoil, I., and Hamm, H. E. (1986) Visual transduction in vertebrate photoreceptors: Light activation of guanylate cyclase, *Cell Biophys. 14*, 129−137.
33. Wolbring, G., Baehr, W., Palczewski, K., and Schnetkamp, P. P. M. (1999) Light inhibition of bovine retinal rod guanylyl cyclase mediated by $\beta\gamma$-transducin, *Biochemistry 38*, 2611−2616.
34. Seno, K., Kishigami, A., Ihara, S., Maeda, T., Bondarenko, V. A., Nishizawa, Y., Usukura, J., Yamazaki, A., and Hayashi, F. (1998) A possible role of RGS9 in phototransduction: A bridge between the cGMP phosphodiesterase system and the guanylyl cyclase system, *J. Biol. Chem. 273*, 22169−22172.
35. Yu, H., Bondarenko, V. A., and Yamazaki, A. (2001) Inhibition of retinal guanylyl cyclase by the RGS9-1 N-terminus, *Biochem. Biophys. Res. Commun. 286*, 12−19.
36. Aparicio, J. G., and Applebury, M. L. (1996) The photoreceptor guanylate cyclase is an autophosphorylating protein kinase, *J. Biol. Chem. 271*, 27083−27089.
37. Tucker, C. L., Laura, R. P., and Hurley, J. B. (1997) Domain-specific stabilization of photoreceptor membrane guanylyl cyclase by adenine nucleotides and guanylyl cyclase activating proteins (GCAPs), *Biochemistry 36*, 11995−12000.
38. Hayashi, F., Lin, G. Y., Matsumoto, H., and Yamazaki, A. (1991) Phsphatidylinositol-stimulated phosphorylation of an inhibitory subunit of cGMP phosphodiesterase in vertebrate rod photoreceptors, *Proc. Natl. Acad. Sci. U.S.A. 88*, 4333−4337.
39. Schnetkamp, P. P. M. (1986) Sodium−calcium exchange in the outer segments of bovine rod photoreceptors, *J. Physiol. 373*, 25−45.
40. Kojima, D., Oura, T., Hisatomi, O., Tokunaga, F., Fukada, Y., Yoshizawa, T., and Shichida, Y. (1996) Molecular properties of chimerical mutants of gecko blue and bovine rhodopsin, *Biochemistry 35*, 2625−2629.
41. Hayashi, F., Hutson, L. D., Kishigami, A., Nagao, S., and Yamazaki, A. (1993) Preparation and characterization of guanylate cyclase from vertebrate rod photoreceptors, *Methods Neurosci. 15*, 237−247.
42. Margulis, A., Pozdnyakov, N., and Sitaramayya, A. (1996) Activation of bovine photoreceptor guanylate cyclase by S100 proteins, *Biochem. Biophys. Res. Commun. 218*, 243−247.
43. Lowe, D. G., Dizhoor, A. M., Liu, K., Gu, Q., Spencer, M., Laura, R., Lu, L., and Hurley, J. B. (1995) Cloning and expression of a second photoreceptor-specific membrane retina guanylyl cyclase (RetGC), RetGC-2. *Proc. Natl. Acad. Sci. U.S.A. 92*, 5535−5539.
44. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72*, 248−254.
45. Yamazaki, A., Tatsumi, M., Torney, D. C., and Bitensky, M. W. (1987) The GTP-binding protein of rod outer segments. 1. Role of each subunit in the GTP hydrolytic cycle, *J. Biol. Chem. 262*, 9316−9323.
46. Subbaraya, I., Zhao, C., and Duda, T. (2003) Structure and $Ca^{2+}$ regulation of frog photoreceptor guanylate cyclase, ROS-GC1, *Mol. Cell. Biochem. 254*, 9−19.
47. 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) Guanylate-cyclase-inhibitory protein is a frog retinal $Ca^{2+}$-binding protein related to mammalian guanylate-cyclase-activating proteins, *Eur. J. Biochem. 252*, 591−599.
48. McDowell, J. H. (1993) Preparing rod outer segment membranes, regenerating rhodopsin, and determining rhodopsin concentration, *Methods Neurosci. 15*, 123−130.
49. McCabe, S. L., Pelosi, D. M., Tetreault, M., Miri, A., Nguitragool, W., Kovithvathanaphong, P., Mahajan, R., and Zimmerman, A.

L. (2004) All-trans-retinal is a closed-state inhibitor of rod cyclic nucleotide-gated ion channels, *J. Gen. Physiol. 123*, 521−531.
50. Hofmann, K. P., Emeis, D., and Schnetkamp, P. P. M. (1983) Interplay between hydroxylamine, meta rhodopsin II and GTP-binding protein in bovine photoreceptor membranes, *Biochim. Biophys. Acta 725*, 60−70.
51. Robinson, W. E., and Hagins, W. A. (1979) GTP hydrolysis in intact rod outer segments and the transmitter cycle in visual excitation, *Nature 280*, 398−400.
52. Biernbaum, M. S., and Bownds, M. D. (1979) Influence of light and calcium on guanosine-5′-triphosphate in isolated frog rod outer segments, *J. Gen. Physiol. 74*, 649−669.
53. Balasubramanian, N., and Slepak, V. Z. (2003) Light-mediated activation of Rac-1 in photoreceptor outer segments, *Curr. Biol. 13*, 1306−1310.
54. Zhang, H., Huang, W., Zhang, H., Zhu, X., Craft, C. M., Baehr, W., and Chen, C.-K. (2003) Light-dependent redistribution of visual arrestins and transducin subunits in mice with defective phototransduction, *Mol. Vision 9*, 231−237.
55. Mendez, A., Lem, J., Simon, M., and Chen, J. (2003) Light-dependent translocation of arrestin in the absence of rhodopsin phosphorylation and transducin signaling, *J. Neurosci. 23*, 3124−3129.
56. Woodruff, M. L., Lem, J., and Fain, G. L. (2004) Early receptor current of wild-type and transducin knockout mice: Photosensitivity and light-induced $Ca^{2+}$ release, *J. Physiol. 557*, 821−828.
57. Janz, J. M., Fay, J. F., and Farrens, D. L. (2003) Stability of dark state rhodopsin is mediated by a conserved ion pair in intradiscal loop E-2, *J. Biol. Chem. 278*, 16982−16991.
58. Doi, T., Molday, R. S., and Khorana, H. G. (1990) Role of the intradiscal domain in rhodopsin assembly and function, *Proc. Natl. Acad. Sci. U.S.A. 87*, 4991−4995.
59. Anukanth, A., and Khorana, H. G. (1994) Structure and function in rhodopsin: Requirements of a specific structure for the intradiscal domain, *J. Biol. Chem. 269*, 19738−19744.
60. Nagata, T., Terakawa, A., Kandori, H., Kojima, D., Shichida, Y., and Maeda, A. (1997) Water and peptide backbone structure in the active center of bovine rhodopsin, *Biochemistry 36*, 6164−6170.
61. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Trong, I. L., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Crystal structure of rhodopsin: A G protein-coupled receptor, *Science 289*, 739−745.
62. Okada, T., Fujiyoshi, Y., Silow, M., Navarro, J., Landau, E. M., and Shichida, Y. (2002) Functional role of internal water molecules in rhodopsin revealed by X-ray crystallography, *Proc. Natl. Acad. Sci. U.S.A. 99*, 5982−5987.
63. Furutani, Y., Shichida, Y., and Kandori, H. (2003) Structure changes of water molecules during the phtoactivation processes in bovine rhodopsin, *Biochemistry 42*, 9619−9625.

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