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Quantitative aspects of cGMP phosphodiesterase activation
in carp rods and cones

(コイ桿体と錐体とでの cGMP ホスホジエステラーゼの
活性化効率の定量的理解)

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ABBREBIATIONS

R, visual pigment

R*, activated visual pigment

Tr, transducin

Tr*, activated transducin

PDE, cGMP phosphodiesterase

RGS9-1, regulators of G-protein signaling 9-1

GRK, G-protein coupled receptor kinase

GC, guanylate cyclase

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid

DTT, dithiothreitol

GTP γ S, guanosine 5'-O-(3-thiotriphosphate)

HPLC, high performance liquid chromatography

TCA, trichloroacetic acid

EDTA, ethylenediaminetetraacetic acid

MWCO, molecular weight cut-off

CBB, Coomassie Brilliant Blue

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

BSA, bovine serum albumin

Tris, tris(hydroxymethyl)aminomethane

IPTG, isopropyl β -D-1-thiogalactopyranoside

SDS, sodium dodecyl sulfate

PMSF, phenylmethylsulfonyl fluoride

GST, glutathione S-transferase

HRP, horseradish peroxidase

ABSTRACT

Cones are less light-sensitive than rods. In our laboratory, it was showed previously in carp that more light (>100-fold) is required in cones than in rods to activate 50 % of cGMP phosphodiesterase (PDE). The lower effectiveness of PDE activation in carp cones is partly due to the fact that the activation rate of transducin (Tr) by light-activated visual pigment (R^*) is 5-fold lower in carp cones than in rods. In this study, I tried to explain the remaining difference. Firstly, I examined the efficiency of activation of PDE by activated transducin (Tr^*). By activating PDE with known concentrations of the active (GTP γ S-bound) form of Tr^* , I found that Tr^* activates PDE at a similar efficiency in rods and cones.

Next, I examined the contribution of R^* and Tr^* lifetimes. In a comparison of PDE activation in the presence (with GTP) and absence (with GTP γ S) of Tr^* inactivation, PDE activation required more light (and was therefore less effective) when Tr^* was inactivated in both rod and cone membranes. This is probably because inactivation of Tr^* shortened the lifetime of Tr^* , thereby reducing the number of activated PDE molecules. The effect of Tr^* inactivation was larger in cones, probably because the lifetime of Tr^* is shorter in cones than in rods. Furthermore, the lifetime of R^* also contributed the effectiveness of PDE activation. From the results, the shorter lifetimes of Tr^* and R^* in cones seem to explain the remaining difference in the effectiveness of PDE activation between rods and cones.

INTRODUCTION

Vertebrates have two types of visual photoreceptors, rods and cones, which convert light-detection signals into electrical signals. As shown in Figure 1A, both rods and cones are consisted of the outer segment and the inner segment containing ellipsoid, myoid, nucleus and synaptic terminal. Molecular mechanism that mediates detection of light and generation of electrical response (light response) in photoreceptor cells is localized in the outer segment, and the inner segment is responsible for the cell metabolism. Rods and cones are distinctive in their morphology of the outer segment.

Rods and cones are different in the properties of light responses (Figs. 1B and C). Figure 1B shows families of flash response evoked by various intensities of light flash in a carp rod (left) and a red-sensitive cone (right). A flash response in cones terminates more quickly than that in rods. As a result, the time resolution of a light stimulus is much higher in cones than in rods. Figure 1C shows a flash intensity-response relation in carp rods and

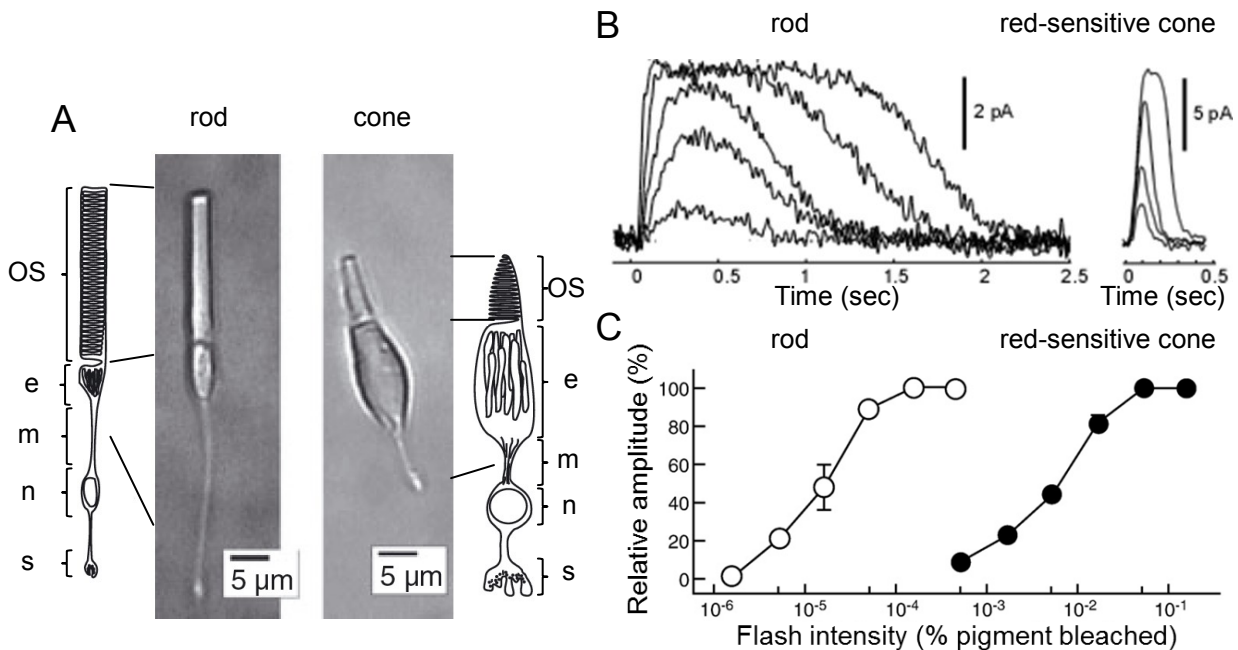


Figure 1. Characterization of Carp Rods and Cones

(A) A mechanically dissociated carp rod (left) and cone (right). The sketch shows the structure of each cell. OS, outer segment; e, ellipsoid; m, myoid; n, nucleus region; s, synaptic terminal region. (B) A flash response family of a rod (left) and a red-sensitive cone (right). Outer segment membrane current was recorded with a suction electrode by giving light flashes of various intensities at time 0 and they are superimposed. The light intensities in a rod used and expressed in the unit of % bleach were (from the dimmest intensity): 5.1×10^{-6} % (corresponding to 8 molecules of visual pigment bleached per rod; 8 R*), 1.6×10^{-5} % (25 R*), 5.1×10^{-5} % (80 R*), 1.6×10^{-4} % (250 R*) and 5.1×10^{-4} % (800 R*). The light intensities in a red-sensitive cone used and expressed in the unit of % bleach were: 1.6×10^{-3} % (2,100 R*), 5.2×10^{-3} % (6,600 R*), 1.6×10^{-2} % (21,000 R*) and 0.16 % (210,000 R*). (C) Light intensity-response relations of rods (*open circles*) and red-sensitive cones (*closed circles*) shown in (B). In (B), the responses were low-pass filtered at 50 Hz. (A) was modified from Figure 1 in Tachibanaki et al. (2007) and (B) and (C) were modified from Figure 1 in Kawamura and Tachibanaki (2008).

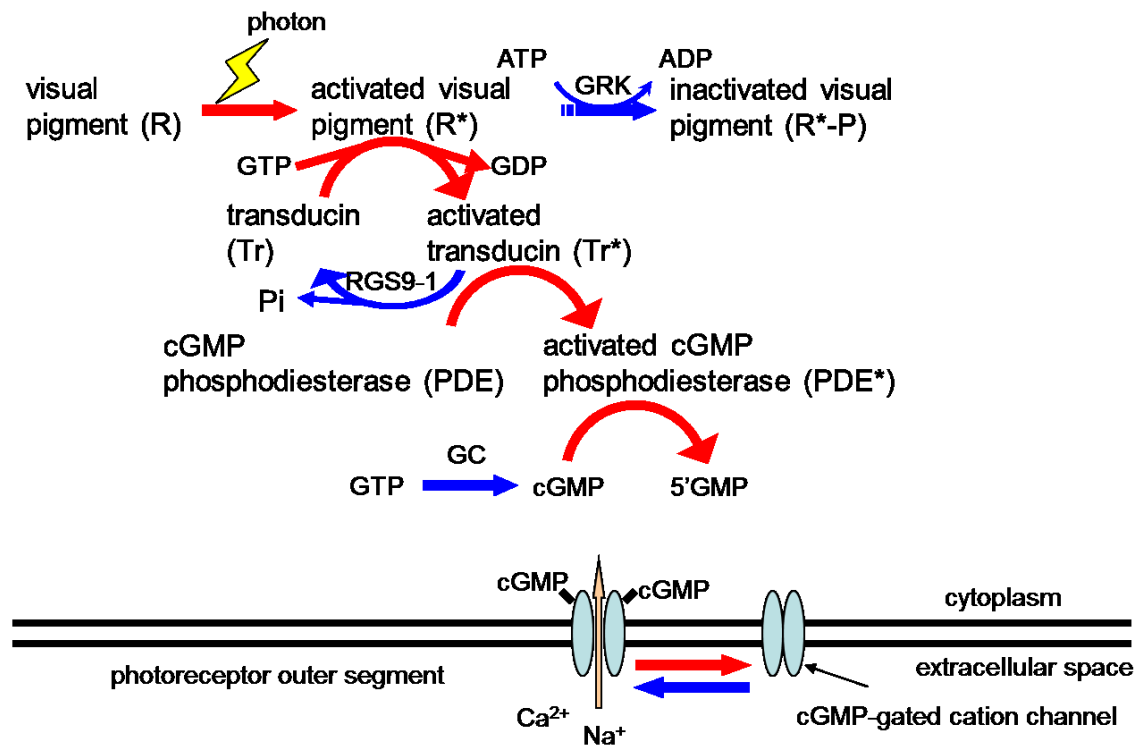


Figure 2. Phototransduction cascade in rods and cones

These reactions occur in the outer segment in rods and cones. Through these reactions, light-detection signals are converted into electrical signals. Red arrows show the reactions responsible for photoresponse generation and blue arrows show the reactions responsible for photoresponse termination. GRK, G-protein coupled receptor kinase; RGS9-1, regulator of G-protein signaling 9-1; GC, guanylate cyclase.

red-sensitive cones. Rods exhibit higher sensitivity to light than cones: in carp, light-sensitivity is >100-fold higher in rods than in cones. Due to this sensitivity difference, rods function in the dark, and cones in the light.

The mechanism that generates light responses is known as the phototransduction cascade (Fig. 2), a process that has been well studied in rods (Pugh and Lamb, 1993; Fu and Yau, 2007; Kawamura and Tachibanaki, 2008). Briefly, visual pigment (R) is activated by absorption of light, and activated visual pigment (R*) catalyzes the exchange of GDP for GTP in a trimeric GTP-binding protein, transducin (Tr). The GTP-bound form of Tr (Tr*) is the active form. Tr* interacts the inhibitory γ subunit of cGMP phosphodiesterase (PDE), and activates PDE (Wensel and Stryer, 1986). Activated PDE hydrolyzes cGMP, resulting in a decrease in the cytoplasmic cGMP concentration. Consequently, cGMP dissociates from a cGMP-gated cation channel situated in the plasma membrane of a rod outer segment; this closes the channel, thereby inducing a hyperpolarizing light response.

After a light stimulus, all activated species (such as R* and Tr*) are inactivated and the decreased level of cGMP is restored. R* is inactivated by phosphorylation and subsequent binding of arrestin (Chen et al., 1999; Xu et al., 1997). Tr* is inactivated by hydrolysis of

bound GTP to GDP via its intrinsic GTPase activity, which is accelerated by a photoreceptor-specific GTPase-accelerating protein, regulator of G-protein signaling 9-1 (RGS9-1) (Cowan et al., 1998). Cytoplasmic cGMP concentration is restored by synthesis from GTP by guanylate cyclase.

In the rod phototransduction cascade, enormous signal amplification takes place. In frog rods, when one molecule of rhodopsin is activated, a total of ~100 molecules of Tr are activated for 1 sec (Leskov et al., 2000). Furthermore, one molecule of PDE catalytic subunit hydrolyzes ~2000 molecules of cGMP for 1 sec (Dumke et al., 1994). These amplification reactions should contribute to high sensitivity to light in rods. In cones, a similar cascade is present, but in many animal species, a different set of players in the cascade (for example, cone-type visual pigment, Tr, PDE and cGMP-gated cation channel instead of their rod-types) is known to be present (Hisatomi and Tokunaga, 2002). It is probable that the efficiencies of the reactions in the cascade, including activation of R and Tr, and inactivation of R* and Tr*, are different between rods and cones, causing the sensitivity and time resolution to differ between rods and cones.

Previously, some of the reactions were compared between rods and cones. The efficiency of visual pigment activation by photon absorption was similar between chicken rhodopsin and iodopsin (red-sensitive cone visual pigment) (Okano et al., 1997). The catalytic activity of a single molecule of PDE was also similar between rod-type and cone-type, both purified from bovine retinas (Gillespie and Beavo, 1988). Moreover, the characteristics of cGMP-gated cation channels in catfish rods and cones were also similar (the Hill coefficient is ~2.3 for both types of channel; Haynes and Stotz, 1997). Thus, some of the reactions were already revealed to be similar between rods and cones.

To understand the mechanisms underlying the differences between the rod and cone light responses quantitatively at the molecular level, a method to purify rods and cones were established about 10 years ago in our laboratory using carp retina (Tachibanaki et al., 2001). This method was the first report of the purification of cones to obtain in quantities large enough to do biochemical studies. The membranes prepared from purified rods and cones are suitable for the study on the reactions in the phototransduction cascade, because the proteins involved in this cascade (e.g. visual pigment, Tr and PDE) are remained in the membranes. Using these membranes, some of the reactions in the phototransduction cascade were compared between rods and cones (Tachibanaki et al., 2001). In that report, to compare the efficiency of PDE activation between rods and cones, PDE activities to light flashes of various intensities were measured in rod and cone membranes and then, the maximum % PDE activity elicited by a light flash (peak PDE activity) was plotted against the flash intensity. In the presence of ATP, and therefore in the presence of R* phosphorylation, the

light intensity required to evoke 50 % of full PDE activity was ~220-fold higher in cones than in rods (by contrast, in this study, the difference was found to be ~40-fold; see “Results and Discussion”). The molar ratio of PDE to R is similar between rods and cones (Tachibanaki et al., 2001 and this study); therefore, the aforementioned requirement of cones for a higher intensity of light means that the efficiency of PDE activation per photon is much lower in cones.

Recently, we reported that this lower effectiveness of PDE activation in cones was partly due to lower activation of Tr by R* (5-fold) in cones than in rods (Tachibanaki et al., 2012). However, the total difference in the effectiveness of PDE activation between rods and cones is not accounted for by this 5-fold difference. Therefore, in this study, I tried to determine which reactions are responsible for the much lower effectiveness of PDE activation in cones relative to rods. Firstly, I compared the efficiency of PDE activation by Tr* between rods and cones. Then I examined the contribution of Tr* and R* lifetimes to peak PDE activity elicited by a light flash to determine how their lifetimes affect the effectiveness of PDE activation in rods and cones.

EXPERIMENTAL PROCEDURES

Preparation of Rods and Cones

Carp (*Cyprinus carpio*: 25-30 cm in length) were purchased from Hirose Carp (Fukushima, Japan). Animal care was conducted according to the institutional guidelines.

Carp rods and cones were isolated as described previously (Tachibanaki et al., 2007) with some modifications. All manipulations were carried out in complete darkness with the aid of an infrared image converter (NVR 2015, NEC) under illumination of >800 nm light by using infrared-emitting diodes (TLN115A, Toshiba) and light bulbs with an infrared transmission filter. Carp were dark-adapted in a light-tight tank covered with a dark curtain for >3 h before use, and the retinas were dissected after sacrificing the animal by pithing during night time. The photoreceptor side of the retina was gently tapped using a paintbrush in a Ringer's solution (4 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid], 16 mM glucose, 119.9 mM NaCl, 2.6 mM KCl, 1 mM NaHCO₃, 0.5 mM CaCl₂, 0.5 mM MgCl₂, 0.5 mM MgSO₄, 0.5 mM NaH₂PO₄, pH 7.5) to mechanically dissociate both rods and cones from the retina, and large fragments of the retina were removed with forceps. The suspension of rods and cones thus obtained was filtered through a nylon mesh (~95 μ m of pore size) to eliminate small fragments of the retinal tissues. Because substantial numbers of rods and cones remained in the removed large and small fragments, rods and cones were collected again by repeating the tapping and filtration. By doing so, the yield of rods and cones was improved; with use of retinal fragments, relative to the numbers obtained in the first dissociation, similar amounts of rods and cones were further recovered maximumly. The filtered sample was layered on the top of a stepwise Percoll gradient (30/45/60/70/75/90 %; w/v) and centrifuged for 20 min at 10,000 \times g at 4°C. The filtered sample from the retinal fragments was separately centrifuged. Rods were sedimented at the 45/60 % interface and cones were sedimented at the 75/90 % interface. These purified rods and cones were collected and mixed with the Ringer's solution to reduce the density of Percoll, and centrifuged (600 \times g for 12 sec and then 3,000 \times g for 4 sec). Purified cone sample thus obtained with this method contained some red blood cells (<50 % of cone cells) so that the sedimented cells were additionally suspended in a potassium-gluconate buffer (K-gluc buffer: 115 mM potassium gluconate, 10 mM HEPES, 2.5 mM KCl, 2 mM MgCl₂, 0.2 mM EGTA [ethylene glycol-bis(2-aminoethyl-ether)-N,N,N',N'-tetraacetic acid], 0.1 mM CaCl₂, 1 mM dithiothreitol [DTT], pH 7.5) and then sedimented again by centrifugation (600 \times g for 12 sec and then 3,000 \times g for 4 sec). With this manipulation, the red blood cells were disrupted and their cytoplasm was removed as the supernatant. In contrast, rods and cones retain their morphology. Although the membrane fractions of red blood cells remained in the cone sample, I found that the red blood cell membranes do not influence the PDE activation. The resultant purified rods and cones were frozen immediately in liquid nitrogen and stored at

-80°C. These purified cells contained their outer segments and ellipsoids, but did not contain the nuclei and synaptic terminals.

Preparation of Rod and Cone Membranes

Purified rods and cones once frozen were thawed and washed 3 times with K-gluc buffer to remove their cytoplasmic fractions (centrifugation for 15 min at $100,000 \times g$ at 4°C). The precipitated membranes were suspended in K-gluc buffer and an aliquot of the suspension was used to quantify the amount of each type of visual pigment in rod and cone membranes. Quantification of each visual pigment was performed by assuming that the molar absorption coefficient is $40,000 \text{ M}^{-1} \text{ cm}^{-1}$ as described previously (Tachibanaki et al., 2001). The membrane suspension thus obtained was stored at -80°C until use.

Light Source

A light flash (Sunpak Auto 25SR) or a 150-W tungsten-halogen lamp was used to bleach visual pigments. In either case, a cut-off filter was used to pass light with wavelengths greater than 410 nm.

Purity of GTP γ S

Commercially available GTP γ S is contaminated with GDP (>20 % of GTP γ S). To quantitate the amount of GTP γ S as accurately as possible, the purity of each sample of purchased GTP γ S was checked by HPLC using a Mono Q column (SMART System, GE Healthcare) (Hartwick and Brown, 1975). Elution was carried out with a linear gradient of KCl starting with buffer Qa (7 mM KH₂PO₄, pH 4.0) and finally with buffer Qb (250 mM KH₂PO₄, 500 mM KCl, pH 4.5). The eluted nucleotides were monitored by the absorbance at 254 nm. The purity of GTP γ S was calculated from each peak area of eluted guanine nucleotides (most abundant nucleotide was regarded as GTP γ S, and other nucleotides were identified with use of standards). The concentration of GTP γ S indicated is its actual calibrated concentration, and that of GDP is also the actual concentration (for both GDP added to the sample and GDP present as a contaminant in the GTP γ S reagent).

Analysis of Nucleotides

During the course of this study, I realized that nucleotides are hydrolyzed in my membrane samples. To know how much ATP or GTP remained in the sample at a certain incubation time, the time courses of ATP and GTP hydrolysis were measured. Each sample (25 μ l) containing rod or cone membranes and various kinds of nucleotides was mixed with an equal volume of 10 % (w/v) trichloroacetic acid (TCA) to quench the GTPase and ATPase activities at a desired time. The sample was centrifuged for 15 min at $20,400 \times g$ at 4°C. A portion (40 μ l) of the supernatant was mixed with 92.4 μ l of 0.13 N NaOH for neutralization. The amount of each nucleotide of the sample was quantified by HPLC using a Mono Q

column or a Mini Q column as described above. The initial rate of the ATP or GTP hydrolysis was determined by fitting the time course with an exponential curve,

$$Y = A - B/k \times (1 - \exp[-k \times t]),$$

where Y is the concentration of a remaining nucleotide (mM), A is the concentration of nucleotide at time 0 (mM), B is the initial rate of nucleotide hydrolysis (mM/sec), k (in sec^{-1}) is a rate constant and t (in sec) is the time after the addition of nucleotides.

Preparation of GTP γ S-bound Form of Rod Transducin

The stably active form of rod Tr with GTP γ S-bound (rTr*-GTP γ S) was used to activate PDE. (GTP γ S or GTP binds to the α subunit of Tr, and the GTP γ S- or GTP-bound form of the α subunit is responsible for the activation of PDE. Here, for simplicity, the abbreviation Tr* was used for indicating the molecular species that activates PDE.) The GTP γ S-bound form of rod Tr (rTr*-GTP γ S) was prepared and purified as described previously (Tachibanaki et al., 1997), with some modifications. Rods purified from 60–100 carp retinas were homogenized and fully bleached for 5 min on ice by using a 150-W tungsten-halogen lamp. Next, the homogenized rod membranes were washed twice with K-gluc buffer. The membranes were further washed with a low-ionic strength buffer (buffer A: 5 mM HEPES, 0.5 mM MgCl₂, 1 mM DTT, pH 7.5) supplemented with 0.2 mM EDTA (ethylenediaminetetraacetic acid), and subsequently washed three times with buffer A. The washed membranes were suspended in buffer A containing 10 μ M GTP γ S to form rTr*-GTP γ S, and then centrifuged to obtain rTr*-GTP γ S in the supernatant. This extraction was repeated three times. Extracted rTr*-GTP γ S was concentrated, and the buffer was replaced with K-gluc buffer by ultrafiltration using a VIVASPIN20 10,000 MWCO filter (GE Healthcare). The amount of rTr*-GTP γ S was quantitated by the Coomassie Brilliant Blue (CBB) staining after SDS-PAGE, using bovine serum albumin (BSA) as a standard. Only three bands, at molecular masses corresponding to those of the Tr α , β and γ subunits, were visible in the CBB staining.

Purification of GTP γ S-bound Form of Rod Transducin α Subunit

From the rTr*-GTP γ S sample prepared as described above, I purified GTP γ S bound form of rod Tr* α subunit (rTr* α -GTP γ S) by using a Blue Sepharose CL6B column (GE Healthcare) as described previously (Heck and Hofmann, 1993) with some modifications. Before loading the sample, the column was equilibrated with buffer B (10 mM HEPES, 5 mM MgCl₂, 1 mM DTT, pH 7.5). The rTr*-GTP γ S sample was filtered using a 0.22 μ m membrane filter, and the filtrate was loaded on the column and proteins in the sample were eluted using a 0–1 M NaCl gradient. The eluted fractions of rTr* α -GTP γ S were detected with SDS-PAGE and concentrated, and the buffer was replaced with K-gluc buffer by ultrafiltration as described above. The amount of rTr* α -GTP γ S was quantitated by CBB

staining after SDS-PAGE, using BSA as a standard.

Quantification of Activated Transducin

When necessary, I quantitated the amount of Tr* by using a [³⁵S]GTPγS filter-binding assay, as described previously (Tachibanaki et al., 2012).

Rod or cone membranes were first suspended in K-gluc buffer supplemented with 150 nM GDP to reduce the binding of GTPγS in the dark (see “Results and Discussion”). This suspension was irradiated for 1 min by using a 150-W tungsten-halogen lamp with neutral density filters (typically, 6–8 % rhodopsin was bleached), and the suspension was mixed with a solution containing [³⁵S]GTPγS, GDP, cGMP, and EGTA to obtain the GTPγS-bound form of Tr (Tr*-GTPγS). cGMP was added to quantitate the amount of Tr* under the same conditions used in the PDE activity measurements (see below). The final sample had a volume of 12 μl and contained 1.5 μM visual pigment, 5 mM cGMP, 0.8 mM EGTA, and 0.6 nM–1.8 μM [³⁵S]GTPγS. GDP was also added at a concentration four times higher than that of GTPγS; this was necessary because in the measurement of the dark control, GTPγS-binding increased significantly in cone membranes when GDP was not added (see “Results and Discussion”). GTPγS-binding in the dark was terminated 60 sec (rods) or 10 sec (cones) after the addition of GTPγS by adding 100 μl of K-gluc buffer containing both 50 mM NH₂OH to inactivate R* and 10 mM cold GTP to terminate the apparent [³⁵S]GTPγS binding. In the light, a similar manipulation was performed to terminate the reaction 60 sec (rods) or 10 sec (cones) after the addition of GTPγS. Separate studies confirmed that GTPγS-binding in the light was completed by these times in both rod and cone membranes. The samples were then filtered through a pre-rinsed nitrocellulose membrane by using a vacuum manifold. The material remaining on the filter membrane was washed three times with K-gluc buffer containing 25 mM MgCl₂ to remove unbound GTPγS, and the nitrocellulose membrane was then dried. The radioactivity of [³⁵S]GTPγS remaining on the membrane was quantitated by using an image analyzer (BAS 2000, Fuji). The manipulations described above were carried out at 20°C.

I also measured the time courses of Tr* activation by R* in rod and cone membranes. A rod or cone membrane suspension in K-gluc buffer (6 μl) was mixed with 6 μl of K-gluc buffer containing GTPγS. The final sample contained 3.0 (rods) or 1.0 (cones) μM visual pigment, 5 mM cGMP, 0.8 mM EGTA, 0.1 or 0.4 mM GDP and 0.1 mM GTPγS labeled with [³⁵S]GTPγS. After pre-incubation for 20 sec, the samples were irradiated by a light flash (Auto 25SR, Sunpak) with neutral density filters and a cut-off filter passing >410-nm light. By this irradiation, 0.027 % (rod) or 0.17 % visual pigment (cone) was bleached. Then, the reaction was quenched by adding 100 μl of K-gluc buffer containing both 50 mM NH₂OH and 10 mM cold GTP at a desired time. The timing of addition was strictly controlled by using a rapid-quench apparatus that could terminate the reaction in less than 0.1 sec

(Tachibanaki et al., 2005). After that, the amounts of bound GTP γ S to Tr were measured as described above. The initial rate of the Tr activation was determined by fitting the results with an exponential curve,

$$Y = A/k \times (1 - \exp[-k \times t]),$$

where Y is the amount of GTP γ S bound to Tr per R* (GTP γ S bound/R*), A is the initial rate of GTP γ S binding (GTP γ S bound/ R*•sec), k (in sec⁻¹) is a rate constant and t (in sec) is the time after a light flash.

PDE Activity Measurement

PDE activity was measured by the pH assay method, as described previously (Tachibanaki et al., 2007). The pH drop caused by hydrolysis of cGMP was monitored with a combination glass microelectrode (MI-410, Microelectrodes). The concentration of cGMP hydrolyzed was calibrated by using the pH drops caused by full hydrolysis of known concentrations of cGMP. The range of pH drops during measurements was less than 0.2 pH units.

Four types of PDE activity measurements were performed under slightly different experimental conditions, as described below. All of the measurements were carried out at room temperature in a V-vial[®] containing 100 μ l of sample.

i) PDE activation by exogenous rod Tr* (rTr*-GTP γ S) or rod Tr* α (rTr* α -GTP γ S)

In the first type of measurements, PDE was activated by exogenous rod Tr* (rTr*-GTP γ S) or rod Tr* α (rTr* α -GTP γ S), prepared as described above. Firstly, rTr*-GTP γ S or rTr* α -GTP γ S was added to rod or cone membranes suspended in K-gluc buffer. One minute after this addition, PDE activity measurement was initiated by adding cGMP (5 mM in final concentration) to suspensions containing (final concentrations) visual pigment (1.5 μ M), EGTA (0.8 mM) and rTr*-GTP γ S (0.025–1.68 μ M) or rTr* α -GTP γ S (0.05–1.4 μ M). PDE activity was determined from the rate of cGMP hydrolysis after the addition of cGMP. Without the addition of rTr*-GTP γ S or rTr* α -GTP γ S, PDE was not activated even in the light. In this type of measurement, the PDE activity was a function of added rTr*-GTP γ S (rTr* α -GTP γ S), so that to obtain the half-maximal concentration of rTr*-GTP γ S, the relation between the amounts of added rTr*-GTP γ S and the PDE activities observed was fitted with a Hill equation,

$$V = V_{max} \times \frac{[S]^n}{[S]^n + [S_{1/2}]^n},$$

where V is the PDE activity expressed as cGMP hydrolyzed per visual pigment present per second (cGMP/R•sec), V_{max} is the maximum PDE activity (cGMP/R•sec), $[S_{1/2}]$ is the

concentration of rTr^* -GTP γ S giving 50 % of the maximum PDE activity (μ M), $[S]$ is the concentration of added rTr^* -GTP γ S (μ M) and n is the Hill coefficient.

ii) PDE activation by endogenous Tr^*

In the second type of measurements, PDE was activated by known concentrations of endogenous Tr^* in rod and cone membranes. Rod or cone membranes suspended in K-gluc buffer were illuminated for 1 min with a continuous light (bleaching 6–8 % of the visual pigment per min), and then GTP γ S was added at various concentrations (0.6 nM–1.8 μ M) ranging from a concentration much lower than that of endogenous rod or cone Tr to a concentration much higher, in order to produce various concentrations of the stably active form of Tr (Tr^* -GTP γ S). When GTP γ S concentration was very low and lower than the concentration of endogenous Tr , the amount of Tr^* -GTP γ S was limited by the amount of added GTP γ S (Bruckert et al., 1994). In other words, in this case, the concentration of Tr^* -GTP γ S did not exceed the concentration of added GTP γ S. The sample was kept in the light for 1 min, and the PDE activity measurement was initiated by adding a solution containing cGMP. The reaction mixture contained 1.5 μ M visual pigment, 5 mM cGMP, 0.8 mM EGTA, 0.6 nM–1.8 μ M GTP γ S (previously added), and GDP as a contaminant at a concentration 0.67 times higher than that of GTP γ S. PDE activity was determined from the rate of cGMP hydrolysis. Full PDE activity elicited by light was measured in a sample of the same batch used for each measurement in the presence of 10 μ M GTP γ S.

iii) PDE activation in the presence of GTP or GTP γ S

In the third type of measurements, PDE activity was compared in the presence of GTP and in the presence of GTP γ S in rod and cone membranes. PDE activity was measured by giving a light flash of various intensities in the presence of 0.1 mM GTP or GTP γ S, and in the presence and absence of ATP. When necessary, the HEPES concentration was reduced (1.5–2.2 mM) to expand pH-drop signals. The reaction mixture contained 0.75 μ M visual pigment, 2.5 mM cGMP, 0.8 mM EGTA, 0.4 mM GDP, 0.1 mM GTP or GTP γ S, and (when necessary) 0.25 mM ATP in K-gluc buffer.

In this type of measurements, the pH drop caused by hydrolysis of ATP was corrected. During incubation with cone membranes, hydrolysis of ATP induced a pH decrease of the sample solution. Because hydrolysis of cGMP was monitored by the pH assay method, the pH decrease caused by ATP hydrolysis recorded in a control measurement was subtracted from the pH record of the measurement of cGMP hydrolysis when ATP was present. To minimize ATP and/or GTP hydrolysis, nucleotides other than cGMP were added 10–15 sec before a light flash. Control studies showed that when the PDE activity reached its peak (1.5–3 sec in cone membranes and 3–8 sec in rod membranes after a light flash), ATP was present at a sufficient concentration (>0.13 mM, Fig. 13; see “Appendix”). In the presence of GTP, the PDE activity gradually increased after a light flash, reached its peak, and then declined to the dark level. The peak amplitude of the PDE activity obtained in the presence of

GTP was a function of the flash intensity, and is referred to as peak PDE activity in the following. When GTP γ S was used, cGMP hydrolysis increased linearly with time, so that this steady PDE activity was determined 10–20 sec after the flash in cone membranes, and 45–55 sec after the flash in rod membranes. Because PDE activity measured in the presence of GTP γ S was also a function of the flash intensity, I also refer to this steady activity as peak PDE activity. Full PDE activity was measured by exposing the sample to light of saturating intensity.

iv) PDE activity measurement with trypsin activated PDE

In the fourth type of measurements, we compared the full PDE activity elicited by a saturating light flash with trypsin-activated PDE activity at various membrane concentrations in rod and cone membranes. In this measurement, we examined the effect on the PDE activity of membrane concentration-dependent elution of Tr from the membranes. Trypsin is thought to digest the PDE inhibitory subunit to relief the inhibitory constraint on the catalytic subunit so that the activity measured with trypsin-activated PDE is of the activity of all PDE molecules present in the sample.

For the activity measurement of trypsin-activated PDE, rod or cone membranes were suspended in K-gluc buffer containing 0.75-15 (rods) or 0.75-1.5 (cones) μ M visual pigment, 0.8 mM EGTA, and 0.039-0.31 mg/ml trypsin, and incubated for 5 min. The time of trypsin treatment and the trypsin concentration were optimized for each membrane concentration. The activity measurement was initiated by addition of 5 mM cGMP. For the activity measurement of the full PDE activity elicited by a saturating light flash, rod or cone membranes were suspended in K-gluc buffer containing 0.75-15 (rods) or 0.75-1.5 (cones) μ M visual pigment, 0.8 mM EGTA, 5 mM cGMP, 0.1 mM GDP and 0.1 mM GTP γ S. The measurement was initiated by giving a saturating light flash bleaching ~10 % of the visual pigment.

Elution of Activated Transducin in Rod Membranes

Membrane concentration-dependent elution of Tr*-GTP γ S was measured in rod membranes with SDS-PAGE. Rod membranes were suspended in 5-100 μ l of K-gluc buffer by varying the pigment (membrane) concentrations. The sample contained 0.75-15 μ M visual pigment (75 pmol), 0.1 mM GDP, 0.1 mM GTP γ S and 0.8 mM EGTA. The samples were fully bleached for 2 min on ice by using a 150-W tungsten-halogen lamp, then centrifuged for 15 min at $100,000 \times g$ at 4°C. The supernatant fraction was mixed with 10 % (w/v) TCA to precipitate all proteins. To reduce the loss of the precipitate through the following centrifugation, each sample volume was adjusted to 120 μ l. If the sample volume exceeded 200 μ l, the yield of precipitated protein decreased. The samples were incubated on ice for 30 min and centrifuged for 15 min at $20,400 \times g$ at 20°C. The precipitate was washed with 500 μ l of K-gluc buffer and suspended in 2 \times sample buffer (1 \times sample buffer: 10 % [w/v] glycerol,

2 % [w/v] SDS, 0.1 % [w/v] Bromo Phenol Blue, 0.05 mM Tris, 1.25 % [v/v] 2-mercaptoethanol, pH 6.8), and applied for SDS-PAGE. Because the band of visual pigment overlaps with the band of Tr α subunit to interfere with the detection of this band, the sample was incubated for 5 min at 95°C to eliminate the visual pigment band by multiple aggregation.

Phosphorylation Assay in Rod Membranes

Phosphorylation assay was performed as described previously (Tachibanaki et al., 2007) with some modifications. Rod membranes were mixed with K-gluc buffer containing [γ - 32 P]ATP, GTP and EGTA. The sample contained 0.75-15 μ M visual pigment, 1 mM [γ - 32 P]ATP, 0.5 mM GTP and 0.8 mM EGTA in K-gluc buffer. After pre-incubation for 20 sec, the sample was irradiated with a light flash (~75 % visual pigment was bleached). The reaction was terminated by adding 10 % (w/v) TCA. The sample was incubated on ice for 30 min and centrifuged for 15 min at $20,400 \times g$ at 20°C. The precipitate was washed with 500 μ l of K-gluc buffer and suspended in 2 \times sample buffer for SDS-PAGE. The amount of 32 P incorporated into the visual pigment band was quantified by an image analyzer (BAS 2000, Fuji). Dark activities, which were almost negligible, were always subtracted to determine light-dependent incorporation of 32 P. Phosphorylation of the visual pigment is expressed in units of the number of phosphates incorporated per visual pigment bleached (Pi/R*). The initial rate of the phosphorylation was determined by fitting the phosphorylation signals with an exponential curve,

$$Y = A/k \times (1 - \exp[-k \times t]),$$

where Y is the number of phosphates incorporated per visual pigment bleached (Pi/R*), A is the initial rate of visual pigment phosphorylation (Pi/ R*•sec), k (in sec $^{-1}$) is a rate constant and t (in sec) is the time after a light flash.

Construction, Expression and Purification of Recombinant PDE6Gs and PDE6H

To quantitate the amount of PDE γ subunits in rod and cone membranes by immunoblot, I expressed recombinant PDE γ subunits in *E.coli* as standard proteins. All clones were obtained from carp retinal cDNA library constructed previously (Shimauchi-Matsukawa et al., 2005). This cDNA library was constructed by using ZAP-cDNA Synthesis Kit (STRATAGENE), therefore, cDNAs were inserted between the EcoRI and XhoI restriction sites of pBluescript SK- vectors. The full lengths of the DNA sequences of carp PDE γ subunits are already known. PDE6G1 (GenBank accession number AB180754) and PDE6G2 (AB180755) were deposited as a nucleotide sequence of rod-type PDE γ . PDE6H (AB180756) was deposited as a nucleotide sequence of cone-type PDE γ . Whole coding region of each carp PDE γ sequence was inserted into the *Nde*I/*Bam*HI sites of

pET 3a expression vector (Novagen).

Expression and purification of PDE γ were performed as described previously (Artemyev et al., 1998) with some modification. BL21(DE3) cells carrying each pET 3a-PDE γ plasmid were grown at 37°C overnight in 3 ml of LB medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) containing 50 μ g/ml ampicillin. The overnight culture was added to one liter of bacterial culture (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl) with 50 μ g/ml ampicillin in an environmental shaker at 37°C until its OD₆₀₀ reaches to 0.8. Subsequently, 0.7 ml of 1 M IPTG was added and incubated for 3 h at 30°C. Cells were collected as a pellet by centrifugation at $2,500 \times g$ for 10 min at 4°C, and washed two times with 100 ml of Wash buffer (50 mM Tris-HCl, 20 mM NaCl, 5 mM EDTA, 1 mM DTT, pH7.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The pellet was freeze-thawed and resuspended in 40 ml of Wash buffer containing 1 mM PMSF. The suspension was sonicated 8 times for 30 sec (UD200, TOMY), followed by centrifugation at $12,000 \times g$ for 30 min at 4°C.

The supernatant containing PDE γ was first loaded on a CM Sepharose Fast Flow column (GE healthcare) equilibrated with Wash buffer. The bound proteins were eluted at a flow rate of 0.5 ml/min using a 20-400 mM NaCl gradient. The fractions containing PDE γ were next purified by reverse-phase HPLC chromatography on a C₁₈ column (5C₁₈-AR, COSMOSIL) with a 0-80 % gradient of acetonitrile in 0.1 % trifluoroacetic acid/H₂O. Purified PDE γ was then lyophilized, dissolved in 20 mM HEPES buffer (pH 7.5), and stored at -80°C until use.

Preparation of Polyclonal Anti-sera

Anti-PDE6G1, PDE6G2 and PDE6H anti-serum were raised in mice against the glutathione S-transferase (GST)-fused proteins. To obtain the GST-fused PDE γ subunit, whole coding region of each carp PDE γ sequence was inserted into the *EcoRI/SalI* sites of pGEX 5X-1 vector (GE Healthcare).

For expression and purification of GST-fused PDE γ , BL21(DE3) cells carrying each pGEX 5X-1-PDE γ plasmid were grown at 37°C overnight in 3 ml of LB medium containing 50 μ g/ml ampicillin. The overnight culture was added to 500 ml of LB medium containing 50 μ g/ml ampicillin in an environmental shaker at 37°C until its OD₆₀₀ reaches to 0.5-1.0. Subsequently, 0.25-0.5 ml of 1 M IPTG was added and incubated for 6 h at 37°C. Cells were collected as a pellet by centrifugation at $2,500 \times g$ for 10 min at 4°C, and suspended in 50 ml of PBS buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH7.4) containing 1 % (w/v) TritonX-100. The suspension was sonicated 5 times for 1 min (UD200, TOMY), followed by centrifugation at $12,000 \times g$ for 15 min at 4°C.

The supernatant was filtered through a 0.45 μ m filter and loaded on a Glutathione Sepharose 4B column (GE healthcare) equilibrated with PBS buffer. The bound proteins were eluted using an elution buffer (10 mM glutathione in 50 mM Tris-HCl, pH 8.0).

Mice were immunized by intraperitoneal injections of 50-100 μ g of each protein in

Freund's complete adjuvant (Sigma). The immunization was repeated two times at an interval of 2 weeks with injection of the same amount of the protein in Freund's incomplete adjuvant. The mice were bled 6-8 days after the final immunization.

Quantification of PDE6G1 in Rod Membranes by Immunoblot

An expression level of PDE6G1 in rod membranes was determined by immunoblot as described previously (Tachibanaki et al., 2005) using recombinant PDE6G1 as molar standards (see above). The amount of recombinant protein was quantitated by CBB staining after SDS-PAGE, using BSA as a standard.

PDE6G1 was probed immunologically and detected by Chemi-Lumi One L (nacalaitesque) using HRP labeled anti-mouse IgG antibodies as secondary antibodies. The content of a protein was expressed as the ratio to that of visual pigment.

RESULTS and DISCUSSION

PDE Activation by Exogenous Rod Transducin in Rod and Cone Membranes

To examine the activation efficiency of PDE by the active form of Tr in rod and cone membranes, firstly, I purified rods and cones from carp retina (Fig. 3). Briefly, rods and cones were brushed off the retina (Tachibanaki et al, 2001; Fig. 3A). The cells were purified by using a stepwise Percoll density gradient. Rods were collected at 45/60 % (w/v) interface (Fig. 3B), and cones were collected at 75-90 % (w/v) interface (Fig. 3C). Purified rods and cones were freeze-thawed and washed with K-gluc buffer to remove the cytoplasmic fractions. The samples were suspended with K-gluc buffer and used as a membrane suspension.

From the purified rods, I purified rod Tr as a GTP γ S-bound form (rTr*-GTP γ S) (Fig. 4A). This purified rTr*-GTP γ S sample contained GTP γ S bound to Tr α subunit (rTr* α -GTP γ S) and Tr $\beta\gamma$ subunits (Figs. 4A and B). Using these samples, I compared the activation of rod and cone PDE by exogenous rod Tr*. To this end, rod PDE in rod membranes and cone PDE in cone membranes were activated by rTr*-GTP γ S at various concentrations.

Figure 4C shows the PDE activity as a function of the concentration of added rTr* α -GTP γ S in rod (*circles*) or cone membranes (*triangles*). Although cone PDE exhibited a

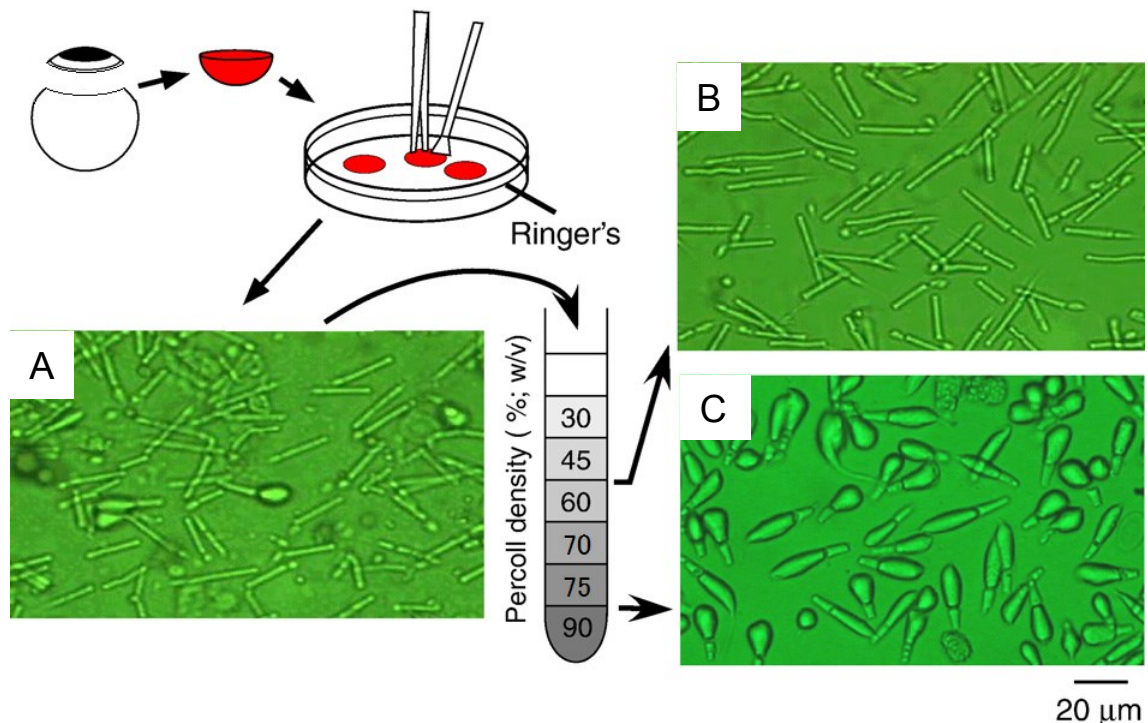


Figure 3. Purification of rods and cones from carp retina

Rods and cones were brushed off the retina. The mixture of rods and cones (A) was layered on the top of a stepwise Percoll gradient. After centrifugation rods were obtained at the 45/60 % interface (B) and cones were obtained at the 75/90 % interface (C). Modified from Figure 3 in Kawamura and Tachibanaki (2008).

slightly higher affinity for rTr* α -GTP γ S than rod PDE, the difference was not significant. The result indicated that rod Tr* activates cone PDE as effectively as it activates rod PDE. The full PDE activities determined in the presence of saturating concentrations of rTr* α -GTP γ S were similar in rod and cone membranes: 15.1 ± 0.6 ($n = 8$) cGMP hydrolyzed per visual pigment present per second (cGMP/R•sec) in rod membranes, and 15.0 ± 0.9 ($n = 6$) cGMP/R•sec in cone membranes. These activities were very similar to that of trypsin-activated PDE in rod membranes (17.4 ± 0.7 cGMP/R•sec, $n = 12$).

Because the specific activities of single molecule of rod and cone PDE are similar

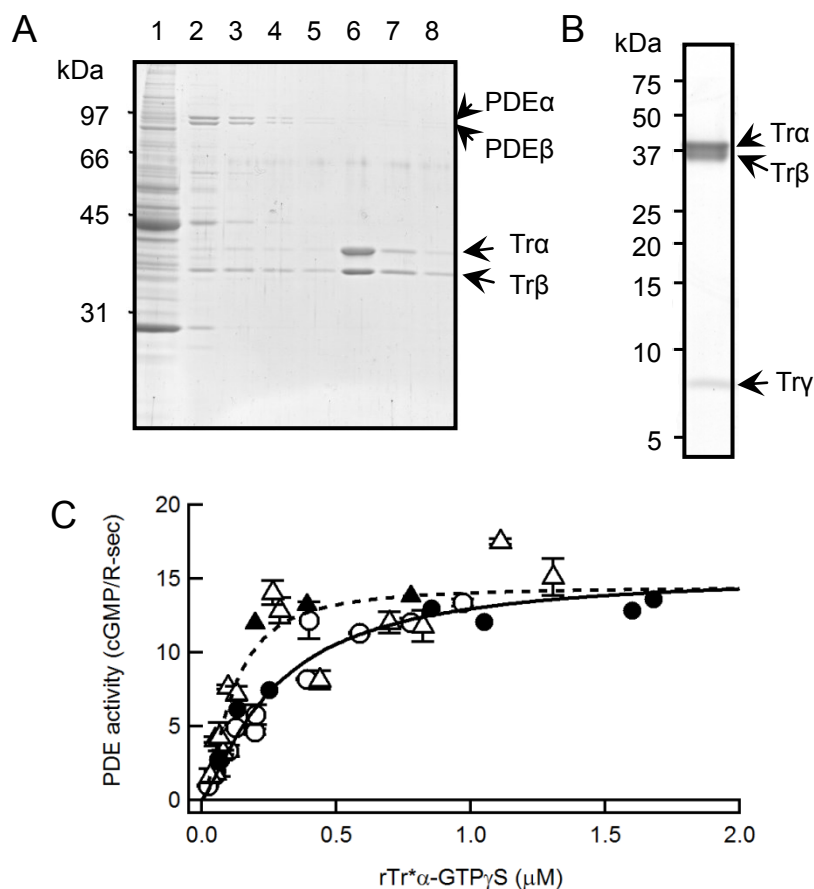


Figure 4. PDE activation by exogenous rod Tr* α -GTP γ S (rTr* α -GTP γ S) in rod and cone membranes (A) rTr* α -GTP γ S purification from carp rods. Each lane was the supernatant of washed rod membranes. Rods were firstly washed with K-gluc buffer (lane 1) and then washed with buffer A containing EDTA (lane 2). The membranes subsequently washed three times with buffer A (lane 3-5). The membranes further washed three times with buffer A containing GTP γ S (lane 6-8). Samples were applied to 12.5 % acrylamide gel and electrophoresed and the gel was stained with CBB R-250. PDE α and β subunits were eluted by washing with buffer A (lane 2-4) and Tr α and β subunits were eluted by washing with buffer A containing GTP γ S (lane 6-8). (B) Purified rTr* α -GTP γ S fraction on Tricine SDS-PAGE. Purified rTr* α -GTP γ S contained Tr γ subunit (~8 kDa). (C) PDE activities elicited by exogenously added rTr* α -GTP γ S were measured in rod (circles) and cone (triangles) membranes (1.5 μ M visual pigment). Each data point indicates a value from a single measurement (closed symbols) or a mean value of multiple measurements (open symbols) in the presence of rTr* α -GTP γ S at the indicated concentrations. Error bars show the deviation from the mean ($n = 2$) or S.E. ($n = 3$). The data data points were fitted using the Hill equation. The best fitted V_{max} , Hill coefficient and $[S_{1/2}]$ values were, respectively, 15.4 cGMP/R•sec, 1.27 and 0.27 μ M in rod membranes (solid curve), and 14.5 cGMP/R•sec, 1.60 and 0.11 μ M in cone membranes (dashed curve).

(Gillespie and Beavo, 1988), the similar maximum PDE activities in rod and cone membranes described above indicated that the number of PDE molecules per visual pigment is similar in rod and cone membranes, as shown previously (Tachibanaki et al., 2001).

To examine the effect of Tr $\beta\gamma$ subunits in rTr * -GTP γ S, I purified rTr * α -GTP γ S from rTr * -GTP γ S sample which contained α , β and γ subunits of Tr (Figs. 5A and B). I measured PDE activities elicited by this purified rTr * α -GTP γ S in rod membranes (Fig. 5C). Although PDE activation efficiency by rTr * α -GTP γ S was modestly lower than that by rTr * -GTP γ S, the difference was not significant. Therefore, I considered that the effect of Tr $\beta\gamma$ subunits in rTr * -GTP γ S on the PDE activation was negligible.

The result shown in Figure 4C indicates that the number of PDE molecules activated by a single molecule of exogenous rTr * α -GTP γ S was similar in rod and cone membranes. In other words, this result strongly suggested that rod Tr * activates rod PDE and cone PDE with

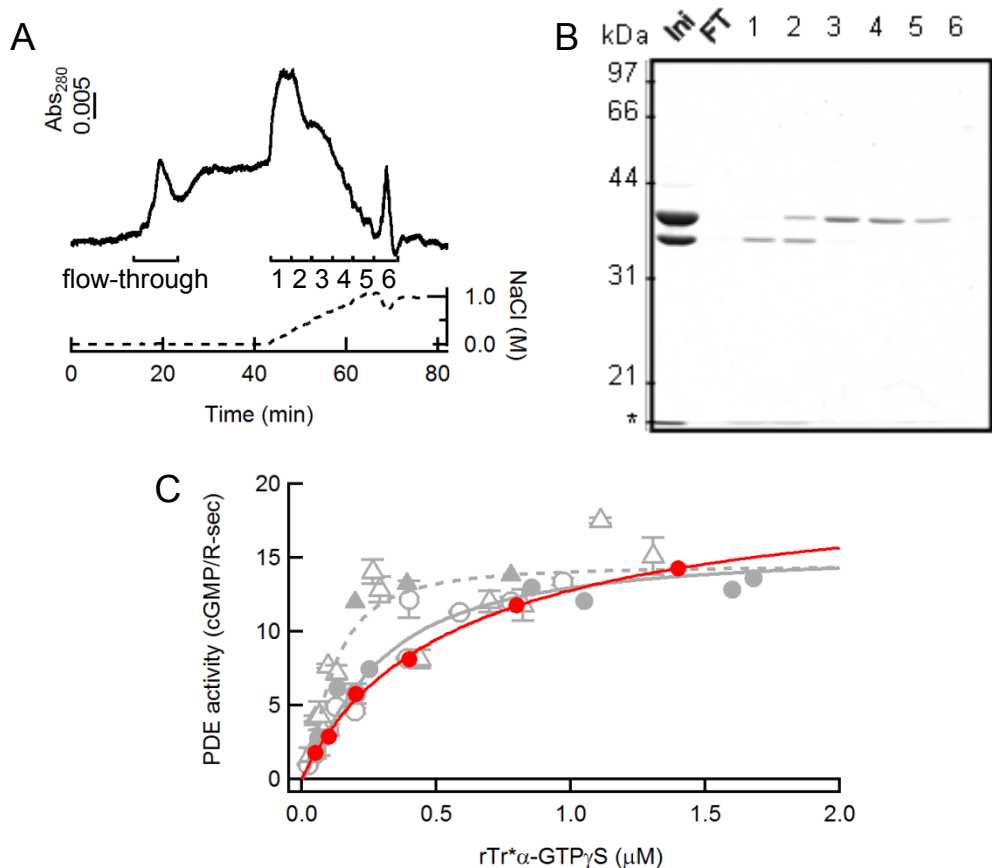


Figure 5. PDE activation by exogenous rod Tr * α -GTP γ S (rTr * α -GTP γ S) in rod membranes (A) rTr * α -GTP γ S purification from rTr * -GTP γ S. Purified rTr * -GTP γ S was loaded onto a Blue Sepharose column. The bound proteins were eluted using a 0-1 M NaCl gradient. (B) SDS-PAGE patterns of the fractions eluted from a Blue Sepharose column. Ini, loaded sample; FT, flow-through fraction; 1-6, eluted fractions using a 0-1 M NaCl gradient. Samples were applied to 12.5 % acrylamide gels and electrophoresed and the gel was stained with CBB R-250. Asterisk indicates dye front. (C) PDE activities elicited by exogenously added rTr * α -GTP γ S (red closed circles, $n = 1$) were measured in rod membranes (1.5 μ M visual pigment). Gray symbols are the data of PDE activities elicited by rTr * α -GTP γ S re-plotted from Fig. 4C. The data points were fitted using a Hill equation. The best fitted V_{max} , Hill coefficient and $[S_{1/2}]$ values were, respectively, 21 cGMP/R·sec, 0.96 and 0.60 μ M in rod membranes (red solid curve).

similar efficiency. Therefore, I next tried to compare the efficiency of activation of rod and cone PDE by endogenous Tr*.

PDE Activation by Endogenous Transducin in Rod and Cone Membranes

To examine the efficiency of PDE activation by Tr* in rod and cone membranes, PDE in rod and cone membranes was activated by known amounts of endogenous Tr*, which was produced by adding limited amounts of GTP γ S in the light. In most of these measurements, the concentration of GTP γ S used was significantly lower than that of endogenous Tr molecules; therefore, under this condition, the number of Tr molecules activated (Tr*-GTP γ S) was limited by the amount of added GTP γ S. Because the sample contained 1.5 μ M visual pigment (R) and the Tr:R ratio was \sim 1:10 (Tachibanaki et al., 2012), the Tr concentration in the sample was estimated to be \sim 150 nM.

Firstly, I calibrated the amount of the GTP γ S-bound form of Tr. Figure 6A shows the relationship between the concentration of GTP γ S added and the estimated concentration of GTP γ S bound to rod (*circles*) and cone (*triangles*) Tr in the dark (*filled symbols*) and in the light (*open symbols*). Saturation in the ordinate at high GTP γ S concentrations is most probably because all GTP γ S molecules bound to Tr. Binding in the dark was very low in rod membranes (*circles* in Fig. 6B, <1 % of the binding in the light), but up to 15 % in cone membranes at the highest concentration (600 nM) of GTP γ S (*triangles* in Fig. 6B). The reason for the high GTP γ S-binding in the dark in cone membranes is not known, but it is possible either that cone visual pigments were thermally activated to induce GTP γ S-binding to Tr in the dark, or that GTP γ S bound to proteins other than Tr. However, it was evident that most of the added GTP γ S bound to rod or cone Tr in the light, because the measured binding curves fitted well to the theoretical curve representing 100 % binding of added GTP γ S (*dashed blue curve* in Fig. 6A).

In Figure 6A, I estimated the total Tr*-GTP γ S concentration at various concentrations of added GTP γ S. Bearing this estimation of the Tr*-GTP γ S concentration in mind, I measured the PDE activity by varying the concentration of GTP γ S. Figures 6C and D show the sample traces of measurements of cGMP hydrolysis in rod (C) and cone (D) membranes in the absence (0 nM) and presence (18 nM or 600 nM) of GTP γ S in the light. Tr*-GTP γ S was produced before the addition of cGMP (see “Experimental Procedures”). Upon the addition of cGMP (*arrows*), the measurement was perturbed; several seconds after the addition, the traces of cGMP hydrolysis became linear. The rate of cGMP hydrolysis (PDE activity) was determined in this linear phase.

It was reported previously that some Tr* molecules were eluted from the membranes (Heck and Hofmann, 2001; Arshavsky et al., 2002). It was possible that eluted Tr* was less effective to activate PDE bound to the membrane. Accordingly, I examined how many Tr* molecules were eluted, and found that at low pigment concentrations I used, a significant proportion of Tr* was eluted from the membranes in rods (see Fig. 10 in “Appendix”).

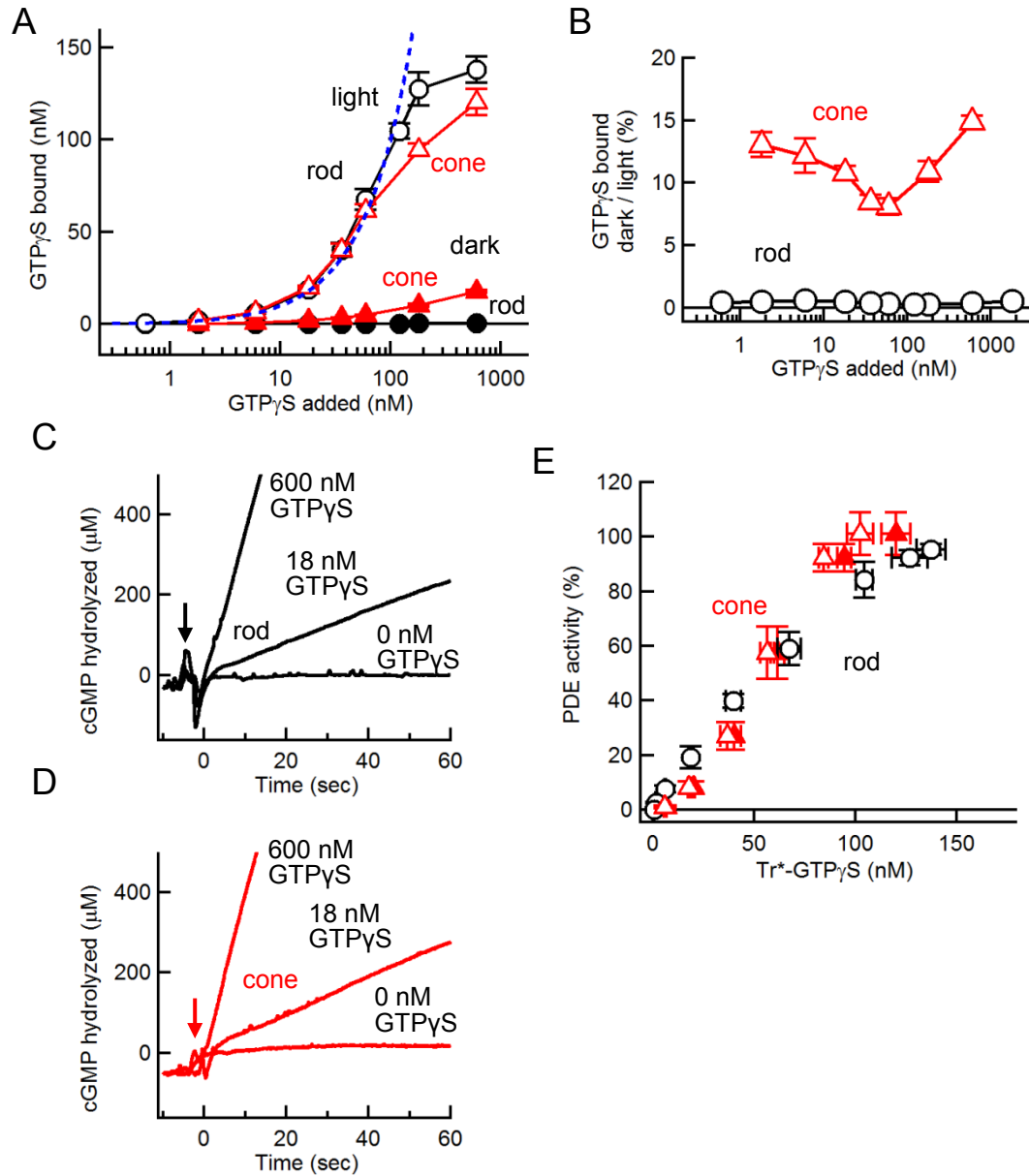


Figure 6. PDE activation by stably active (GTP γ S-bound form) endogenous Tr in rod and cone membranes

(A) Estimated concentrations of GTP γ S-bound form of Tr in the sample expressed as a function of added GTP γ S in the light (*open symbols*) and dark (*filled symbols*), in rod (*circles*) and cone (*triangles*) membranes. Rod or cone membranes were illuminated throughout the measurement with a light that bleached 6–8 % of rhodopsin per min, and GTP γ S was added to the membranes 1 min after the onset of light. A theoretical curve showing 100 % GTP γ S-binding is indicated by a *dashed blue curve*. (B) The ratio of GTP γ S-binding in the dark to that in the light in rod (*circles*) and cone (*triangles*) membranes. The data was calculated from (A). (C and D) Sample traces of PDE activity measurement by the pH assay method. *Arrows* indicate the addition of cGMP. GTP γ S concentrations used in the sample traces are indicated. (E) Relative PDE activity as a function of the concentration of estimated Tr*-GTP γ S. PDE activity, relative to its full activity determined from traces like those shown in (C and D), was plotted against the Tr*-GTP γ S concentration estimated in (A) in rod (*open circles*) and cone (*red triangles*) membranes. In the case of cone membranes, the concentration of Tr*-GTP γ S was estimated by assuming that the total binding of GTP γ S measured in the light reflects the amount of Tr*-GTP γ S (*filled red triangles*) or that the difference in binding between light and dark reflects the amount of Tr*-GTP γ S (*open red triangles*). In (A), (B) and (E), each data point indicates mean \pm S.E. ($n = 3$).

Although I could not detect the elution of Tr* in cone membranes, the reduction in the light-induced PDE activity, probably due to the elution of Tr*, was similar in rod and cone membranes at 0.75-1.5 μ M pigment, the concentration I used (Fig. 11 in “Appendix”). For this reason, I believe that the extent of Tr* elution would have been similar between rod and cone membranes.

Based on the estimation of Tr*-GTP γ S concentration in Figure 6A, I plotted PDE activities as a function of total Tr*-GTP γ S (Fig. 6E). Because I was not certain whether the GTP γ S-binding in cone membranes in the dark represented binding to Tr or to other proteins, the amount of Tr*-GTP γ S in cone membranes was estimated in both ways: *closed triangles* are taken from the total GTP γ S-binding in the light, and *open triangles* from the binding calculated from the light-dark differences in Figure 6A. Because GTP γ S-binding in rod membranes in the dark was almost negligible, only the binding in the light is shown in Figure 6E (rod data; *open black circles*). The full PDE activity elicited by a light flash varied slightly in each measurement, so that the PDE activity expressed on the ordinate was normalized to the full activity in each measurement, but the average of the activity in rod membranes was similar to that in cone membranes. It is evident from Figure 6E that the efficiency of PDE activation, and therefore the number of PDE molecules activated by a single molecule of endogenous Tr*-GTP γ S, was similar between rods and cones. In other words, the photon-capture signal is transmitted to PDE from Tr at a very similar efficiency between rods and cones in the carp retina.

Contribution of Tr Lifetime to PDE Activity*

The experiments described in the previous section demonstrated that each molecule of Tr* activates PDE at a similar efficiency in rods and cones. However, those experiments were performed with a stably active form of Tr (Tr*-GTP γ S). In the actual situation in living cells, Tr* molecules accumulate with time as long as the visual pigment is active, and each Tr* molecule has its own lifetime. For this reason, the maximum number of PDE molecules activated, and thus the peak PDE activity elicited by a weak light flash, could be a function of the lifetimes of individual Tr* molecules activated and inactivated at different times after the light flash. In our previous study, we found that the lifetime of Tr* was 25-fold shorter in cones than in rods, probably due to higher expression of RGS9-1 in cones (Tachibanaki et al., 2012). Although the activation efficiency of PDE by Tr* could be almost the same in rods and cones, as shown in Figure 6, the number of PDE molecules activated by the same number of Tr* molecules could be lower in cones than in rods under conditions in which Tr* is inactivated.

To test this possibility, I compared peak PDE activities by giving a light flash under two different conditions: with GTP γ S, in the absence of Tr* inactivation, and with GTP, in the presence of Tr* inactivation. I assumed that the amounts of Tr* remaining in, and eluted from, the membranes were similar between rod and cone membranes.

There were two problems in this measurement (see “Appendix”). One problem was a considerable PDE activation in the dark in cone membranes (typically, >50 % of full PDE activity was elicited within 1 min after the addition of 0.5 mM GTP). The reason for this increase in PDE activity in the dark was probably because Tr was activated in the dark to some extent (see Fig. 12A in “Appendix”). Based on this idea, I was able to decrease the PDE activation in the dark by increasing GDP concentration and decreasing GTP concentration (see Fig. 12B in “Appendix”).

The other problem is the considerable changes in the concentrations of nucleotides in cone membranes probably mediated by ATPase and GTPase (see “Appendix”). It was probably because purified cones retain large ellipsoid regions (Figs. 1A and 3C) that contain the hydrolytic enzyme(s) for ATP and GTP. To minimize the changes in the nucleotides concentration, I completed each measurement as quickly as possible: in cone membranes, nucleotides except cGMP were added 10-15 sec before giving a light flash. I verified that with HPLC, the changes of the nucleotides concentration were small, and that these changes do not affect the reactions in the phototransduction cascade (see Figs. 13 and 14 in “Appendix”).

Based on the results described above, by giving a light flash, I compared PDE activities in the presence of GTP and those in the presence of GTP γ S. Figures 7A and B show sample traces of PDE activity measurements by the pH assay method in the presence of ATP, in rod (A) and cone (B) membranes, in the presence of GTP (*thick red traces*) or GTP γ S (*thin red traces*). In Figure 7A, a light flash bleaching 0.024 % of the visual pigment in rod membranes was given at time 0, and in Figure 7B, a light flash bleaching 0.46 % of the visual pigment in cone membranes was given. First derivatives were extracted from these traces, and the time courses of PDE activity were determined (Figs. 7C and D; obtained from Figs 7A and B, respectively). In the presence of GTP γ S, PDE activity increased monotonically to a steady level (*thin red traces* in Figs. 7C and D), whereas in the presence of GTP, PDE activity reached a peak and then declined to the dark level (*thick red traces* in Figs. 7C and D). The pH traces were noisy, especially in the case of cone membranes (Fig. 7D). Therefore, I approximated peak PDE activities by eye (*horizontal dashed blue lines* in Figs. 7C and D).

The measurements were made with light flashes of various intensities, and the peak activity relative to the full activity, i.e., the percentage of PDE molecules activated in the presence of ATP, was plotted against the flash intensity (Fig. 7E). The effectiveness of PDE activation by light was higher in the presence of GTP γ S (*filled symbols*) than in the presence of GTP (*open symbols*) both in rod (*circles*) and cone (*triangles*) membranes. This result indicated that Tr* lifetime affects the number of PDE molecules activated by a light flash. However, as shown in Figure 7E, in the case of rods, this effect was not so large, and the difference was almost negligible. By contrast, in cones, the effectiveness of PDE activation by light was decreased by approximately 2-fold in the presence of GTP relative to the presence of GTP γ S. This larger difference in cones may be due to the shorter lifetime of Tr*

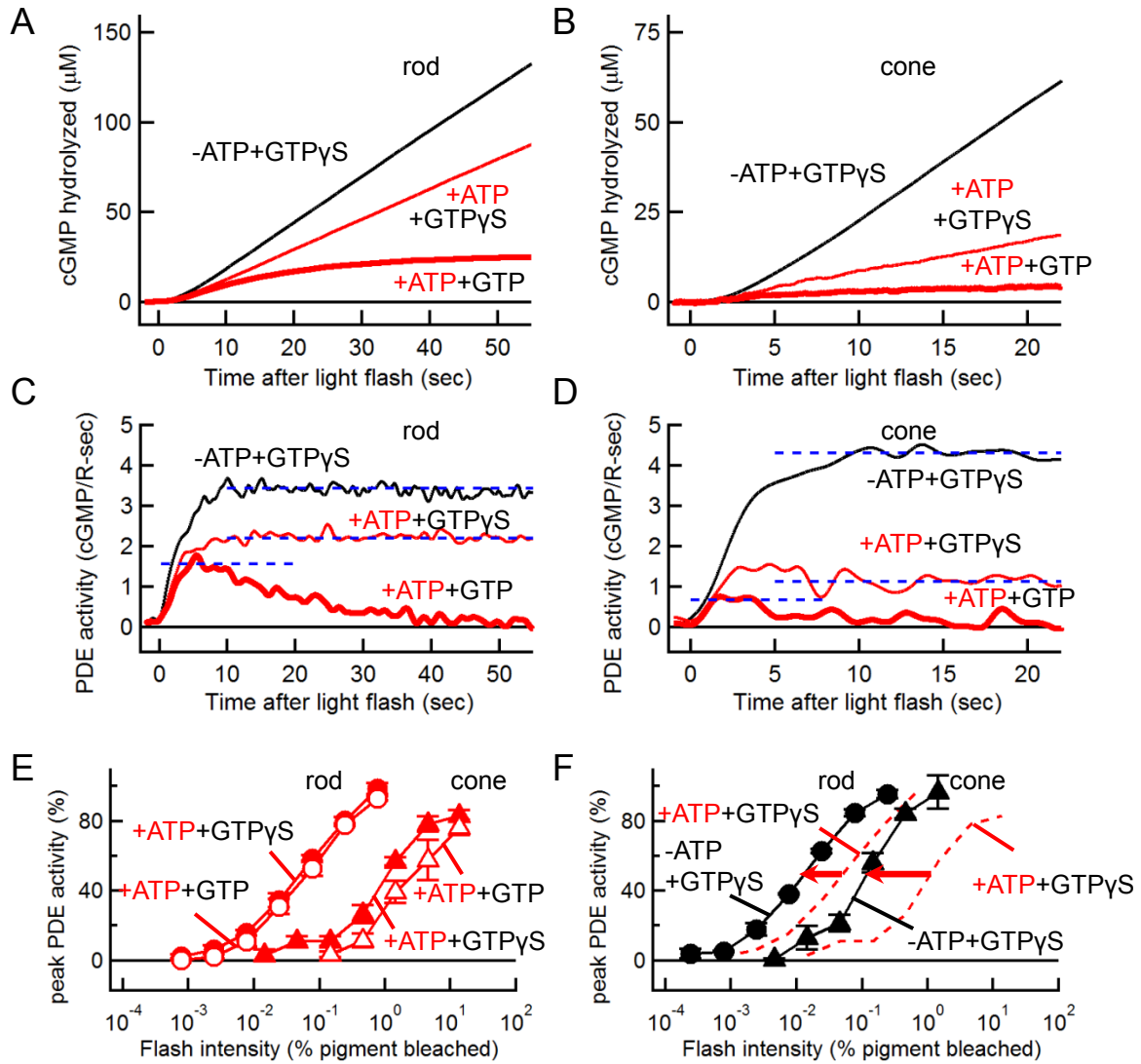


Figure 7. Contributions of Tr^* and R^* lifetimes to PDE activity

(A) Sample traces of PDE activity measurement by the pH assay method in rod membranes. A light flash bleaching 0.024 % of the visual pigment was given at time 0 in the presence of ATP (red traces) and either GTP (thick trace) or GTP γ S (thin trace). Measurements were also made in the absence of ATP with GTP γ S present (black trace). (B) PDE activity measurement in cone membranes. As in (A), a light flash bleaching 0.46 % of the visual pigment was given at time 0 in the presence of ATP (red traces) with either GTP (thick trace) or GTP γ S (thin trace), or in the absence of ATP with GTP γ S (black trace). (C and D) PDE activation and inactivation time courses. PDE activities in rod membranes (C) were determined from the first derivatives of the traces in (A), and those in cone membranes (D) were determined from (B). Peak PDE activity was determined by eye (dashed blue lines). (E) Contribution of Tr^* inactivation (GTP hydrolysis) on peak PDE activity in the presence of ATP in rod and cone membranes. PDE activity measurements in the presence of ATP (see sample red traces in A–D) were made at various intensities of light flash, and the relationship between flash intensity and peak PDE activity was plotted for the results obtained in the presence of GTP γ S (filled symbols) or GTP (open symbols) in rod (circles) and cone (triangles) membranes. (F) PDE activity measurements in the absence of ATP with GTP γ S present (see sample black traces in A–D) were also made at various intensities of light flash, and the relationship between flash intensity and peak PDE activity was plotted for rod (circles) and cone (triangles) membranes. The results of PDE activity measurements in the presence of ATP and GTP γ S are re-plotted from (E) (dashed lines): to avoid crowding, only the connected lines are shown. In all experiments, the sample contained 0.75 μM visual pigment. Arrows show the increase in the effectiveness of PDE activation in the absence of R^* phosphorylation. In (E and F), the results are shown as means \pm S.E. ($n = 3$).

in cones than in rods, as predicted above. Thus, shorter Tr* lifetime in cone membranes contributes a 2-fold reduction in the effectiveness of PDE activation.

In Figure 7E, the difference in the effectiveness of PDE activation in the presence of both ATP and GTP γ S between rod and cone membranes (*filled circles* and *filled triangles*) reflects the difference in the number of Tr* molecules activated by R* during its lifetime, because Tr* is not inactivated in the presence of GTP γ S. This difference, therefore, could be due to the multiplied effect of the differences in both the activation of Tr by R* and the lifetime of R* in the presence of ATP. The overall difference was 21-fold (*filled circles* and *filled triangles*) and the Tr activation rate is 5-fold lower in cone membranes than in rod membranes (Tachibanaki et al., 2012). When this lower activation efficiency of Tr by R* is taken into account, I can predict that in the presence of R* phosphorylation, the lifetime of R* is shorter in cones than in rods, and that the shorter lifetime of R* in cones reduces the effectiveness of PDE activation by approximately 4-fold (21/5). The effectiveness of PDE activation in the presence of both ATP and GTP was 42-fold lower in cones than in rods (*open circles* and *open triangles*), and this difference was somewhat smaller than the value reported previously (220-fold). I believe that this difference is due to the difference in the concentration of GDP (see below).

Contribution of R Phosphorylation on PDE Activation*

The contribution of the lifetime of R*, described above, was estimated in the presence of R* phosphorylation. Therefore, it was of interest to determine how R* phosphorylation contributes to PDE activation. To estimate this contribution, I also measured peak PDE activities in the absence of ATP in rod and cone membranes in the presence of GTP γ S and thus in the absence of Tr* inactivation (*black traces* in Figs. 7A–D). The measurements were made at various intensities of light flashes. In Figure 7F, the relationship between flash intensity and peak PDE activity was plotted for rod (*black circles*) and cone membranes (*black triangles*), and these data were compared with the results obtained in the presence of both ATP and GTP γ S shown in (E) (*dashed lines*, re-plotted from connected lines in E). Because peak PDE activities were measured in the presence of GTP γ S in these studies, the difference in the effectiveness of PDE activation should be due to the difference in the amount of Tr* produced during the lifetime of R* in the presence (+ATP) and absence (-ATP) of R* phosphorylation. In the absence of R* phosphorylation, relative to its presence, the effectiveness of PDE activation increased 3.8-fold in rod membranes and 9.5-fold in cone membranes (*arrows*). Apart from the fact that the lifetime of a bleaching intermediate is shorter in cones than in rods in living cells (Golobokova and Govardovskii, 2006), the effectiveness of R* phosphorylation was 2.4-fold (9.5/3.8) larger in cone membranes.

R* phosphorylation is catalyzed by G-protein coupled receptor kinase (GRK) (Kawamura and Tachibanaki, 2008). GRK is a peripheral membrane protein and localizes in the membrane fraction when rod or cone membranes are centrifuged (Tachibanaki et al.,

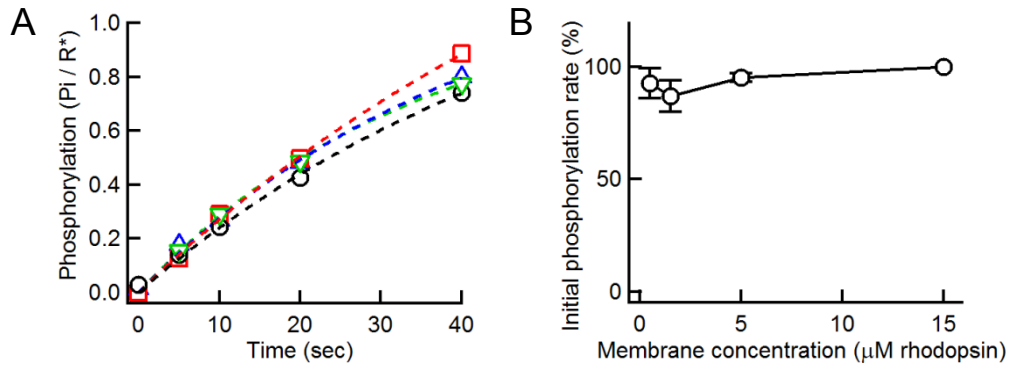


Figure 8. Visual pigment phosphorylation at various rod membrane concentrations

(A) Visual pigment phosphorylation time courses at various membrane concentrations in rod membranes. Visual pigment phosphorylation time courses were measured at 0.75-15 μM visual pigment concentrations by giving a light flash bleaching 75 % of the visual pigment. The results at 0.75, 1.5, 5.0 and 15 μM visual pigment concentrations are shown as *black circles*, *red squares*, *blue triangles* and *green inverted triangles*, respectively. Each sample contained 1 mM ATP. The data points were fitted using an exponential curve ($Y=A/k \times [1-\exp\{-k \times t\}]$). The best fitted A and k values were, respectively, 0.026 $\text{Pi/R}^* \cdot \text{sec}$ and 0.019 sec^{-1} at 0.75 μM visual pigment (*black dashed curve*), 0.029 $\text{Pi/R}^* \cdot \text{sec}$ and 0.014 sec^{-1} at 1.5 μM visual pigment (*red dashed curve*), 0.031 $\text{Pi/R}^* \cdot \text{sec}$ and 0.024 sec^{-1} at 5.0 μM visual pigment (*blue dashed curve*) and 0.033 $\text{Pi/R}^* \cdot \text{sec}$ and 0.029 sec^{-1} at 15 μM visual pigment (*green dashed curve*). (B) Initial rates of visual pigment phosphorylation at various membrane concentrations in rod membranes plotted against the membrane concentrations. In each measurement, the initial phosphorylation rate was normalized to the rate at 15 μM visual pigment. Each data point indicates mean \pm S.E. ($n = 3$).

2005). For this, I assumed that the phosphorylation rate was not dependent on the membrane concentration, but just to be sure, I examine the membrane concentration dependency of the phosphorylation rate in rod membranes (Fig. 8). In Figure 8A, the phosphorylation time course was measured in the presence of excess amount of R^* (75 % visual pigment was bleached). From the result, the initial rate was obtained by fitting the phosphorylation data with an exponential curve (Fig. 8B). At the membrane concentration of 0.75 μM visual pigment, which I used in my study (Fig. 7), the initial rate was similar to that at the highest membrane concentration examined (15 μM visual pigment). Therefore, I concluded that the phosphorylation rate was not dependent on the membrane concentration.

In summary, under a pseudo-intracellular condition in which R^* phosphorylation is present, the difference in the effectiveness of PDE activation between rods and cones, determined by peak activities, was found to originate from three steps in the phototransduction cascade. Firstly, at the stage when Tr^* is generated by R^* , the signal amplification is 5-fold lower in cones than in rods (Tachibanaki et al., 2012). Secondly, in the presence of R^* phosphorylation, the shorter lifetime of R^* in cones decreases the effectiveness of PDE activation 4-fold. Thirdly, the shorter lifetime of Tr^* in cones decreases the effectiveness 2-fold. Overall, due to lower amplification and shorter lifetimes of R^* and Tr^* , the effectiveness of PDE activation was lowered in cones to $\sim 1/40$ ($1/5 \times 1/4 \times 1/2$; multiplied effect of lower Tr^* activation, faster R^* phosphorylation and faster Tr^*

inactivation) of the effectiveness in rods.

Signal Amplification Difference between Rods and Cones in Other Animals

Signal amplification in the phototransduction cascade has been compared between rods and cones previously (Pugh and Lamb, 1993). Pugh and Lamb formulated an equation for the quantitative analysis of signal amplification in the phototransduction cascade. The equation can be applied to a light response to determine an index of the degree of amplification. The index, the amplification constant, can be determined by fitting the initial rise of a light response with the equation. The constants are determined from rods and cones of various animal species. The constant is an inverse function of a cell volume. When this volume factor is taken into account, signal amplification seems to be comparable between mammalian rods and cones but this similarity does not seem to hold in amphibian rods and cones (Pugh and Lamb, 1993). In my study, I showed that the signal amplification at the stage of PDE activation by Tr^* is the same between rods and cones (Fig. 6). In our previous study, signal amplification was 5-fold lower in cones than in rods at the stage of Tr activation by R^* . The hydrolytic rate of a single PDE^* molecule and the number of PDE molecules per visual pigment are very similar between rods and cones (Gillespie and Beavo, 1988; Tachibanaki et al., 2001). From these results we could conclude that the signal amplification is 5-fold lower in carp cones than in carp rods. There is a report that in mice, signal amplification may be lower in cones than in rods (Nikonov et al., 2006). Although further detailed studies are absolutely necessary, it may be the case that the rate of the reaction in the phototransduction cascade is different between rods and cones, and that the extent of this difference depends on the species.

Mechanism of PDE Activation by Transducin in Rods and Cones

Holo-PDE is composed of 4 subunits, i.e. 2 catalytic (α and β) and 2 inhibitory (γ) subunits in rod-type PDE. PDE is activated by displacement of the γ subunit (PDE_γ) from its catalytic subunit (Wensel and Stryer, 1986). The stoichiometry of removal of PDE_γ by Tr^* is 1:1 (Leskov et al., 2000). Because $\text{Tr}^*\text{-GTP}\gamma\text{S}$ activated PDE at similar efficiencies in rods and cones (Fig. 6E), cone Tr^* probably removes PDE_γ of cone PDE at 1:1 stoichiometry.

However, this does not indicate that all Tr^* molecules bind to PDE_γ . As shown in Fig. 6E, maximum PDE activation was observed at ~ 100 nM $\text{Tr}^*\text{-GTP}\gamma\text{S}$ at the 1.5 μM visual pigment concentration. In this measurement, 66 % of $\text{Tr}^*\text{-GTP}\gamma\text{S}$ could have been eluted from the membranes and 34 % of them remained in the membranes (Fig. 10, see “Appendix”). In case the eluted $\text{Tr}^*\text{-GTP}\gamma\text{S}$ does not have activity to activate PDE, ~ 30 nM is the lower limit of the concentration of $\text{Tr}^*\text{-GTP}\gamma\text{S}$ that contributes to activate all of the PDE molecules present in the membranes. This concentration is higher than the concentration of PDE in the membranes; assuming that the molar ratio of holo-PDE to visual pigment is 1:270 in rods (Dumke et al., 1994), at 1.5 μM visual pigment concentration, the holo-PDE concentration is

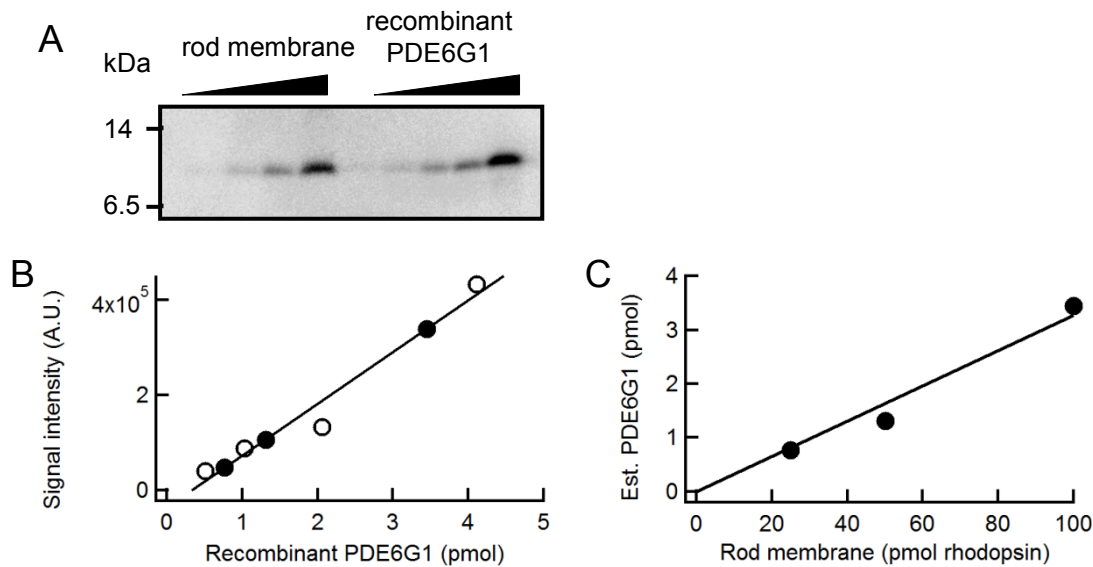


Figure 9. Quantification of PDE6G1 in rod membranes

(A) Quantitative immunoblot of PDE6G1 in rod membranes. Various amounts of rod membranes and purified recombinant PDE6G1 were probed with anti-PDE6G1 antiserum. (B) An example of quantification of PDE6G1. The data were obtained from the result in (A). In the calibration curve obtained by immunoblot of known molar amounts of recombinant PDE6G1 (*open circles*), the signal from rod membrane fractions were plotted (*closed circles*). (C) The calculation of the ratio of PDE6G1 to rhodopsin. The amounts of PDE6G1 estimated from (B) were plotted to the amount of rhodopsin. The ratio was obtained by linear fitting.

estimated to be 5.6 nM or the concentration of the catalytic subunit of PDE to be 11.2 nM. This calculation shows that molar excess of Tr*-GTP γ S is required for activation of PDE. A similar observation was reported in a previous study of rod membranes (Bruckert et al., 1994).

To estimate the amount of holo-PDE with a different method, I tried to quantitate the amount of PDE γ by immunoblot in rod and cone membranes. Unfortunately among the three types of PDE γ , i.e. rod-type PDE γ (PDE6G1 and PDE6G2) and cone-type PDE γ (PDE6H) (see Experimental Procedures), I obtained only anti-PDE6G1 and anti-PDE6G2 antiserum. By immunoblot using these antisera, PDE6G1 was detected in rod membranes, but PDE6G2 was not detected at all. Then, I quantified the amounts of PDE6G1 in rods (Fig. 9). By quantitative immunoblot, I determined the amount of PDE6G1 to be 0.047 ± 0.008 (mean \pm SE, $n = 3$) per rhodopsin present (Fig. 9C). Assuming that the molar ratio of holo-PDE to visual pigment is 1:270 in rods (Dumke et al., 1994), the amount of PDE γ is estimated to be 0.0074 (2/270) per rhodopsin. My value is 6-fold ($0.047/0.0074$) higher than this ratio. PDE γ is exclusively localized to the outer segment of photoreceptor cells as a subunit of holo-PDE (Tsang et al., 2006), and so there should be no contribution from the inner segment. My higher value may suggest that PDE γ is expressed more abundantly than the catalytic subunit of PDE. Alternatively, it is possible that the higher value may be due to underestimation of the amount of the standard protein that was quantitated by CBB staining using BSA as a molar standard. Obviously, further careful study is needed to quantitate the content of PDE in rod and cone membranes.

In this study, the effectiveness of PDE activation was 42-fold lower in cone membranes than in rod membranes in the presence of both ATP and GTP. This difference in the effectiveness of PDE activation was lower than the value reported previously (220-fold; Tachibanaki et al., 2001). The reason for this inconsistency is not clear, but it is possible that in previous study, GTP hydrolysis in cone membranes was much higher than in rod membranes; the resultant GDP, which inhibits Tr activation (Figs. 12 and 14), may have reduced the effectiveness of Tr* production in cone membranes.

Summary

I showed that the efficiency of the PDE activation by Tr* is similar between rods and cones. However, due to the shorter lifetimes of R* and Tr* in cones, PDE activation becomes less effective in cones. Although the quantitative contribution of the reduced effectiveness of PDE activation to the reduced light sensitivity in cones needs to be determined, it is evident that the reduced rate of cGMP hydrolysis tends to shorten the light response time-to-peak, i.e., the time at which the hydrolysis and the synthesis of cGMP are balanced. Because photoreceptor light sensitivity is defined as the amplitude of a peak response, shortened time-to-peak contributes to lower light-sensitivity in cones than in rods.

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APPENDIX

Effect of Tr Elution on PDE Activation in Rod and Cone Membranes*

Tr and PDE are present in the membranes in the dark. When Tr is activated by R*, some Tr* molecules dissociate from the membranes and are found in a soluble fraction (Heck and Hofmann, 2001; Arshavsky et al., 2002). This behavior of Tr molecule is consistent with the finding of the light-dependent translocation of rod Tr from the outer segment to the inner segment (Sokolov et al., 2002). It is possible that eluted Tr* activates PDE less effectively than Tr* remaining in the membranes. To examine this possibility, under the condition used in this study, I measured the elution of Tr* from the rod membranes by SDS-PAGE (Fig. 10). In Figure 10B, a significant proportion of Tr α subunit, and probably $\beta\gamma$ subunits also, were eluted from the rod membranes at low visual pigment concentrations (~65 % and ~50 % elution, or 35 % and 50 % remaining in the membranes at 0.75 μ M and 15 μ M visual pigment, respectively). In cone membranes, it was not possible to detect the elution of cone Tr by SDS-PAGE, because so many bands, probably derived from the cone inner segment, hampered the detection of the Tr α and β bands.

To understand how the elution of Tr* from the membranes affects the PDE activity, I further compared the PDE activity elicited by light of saturating intensity (light-induced PDE

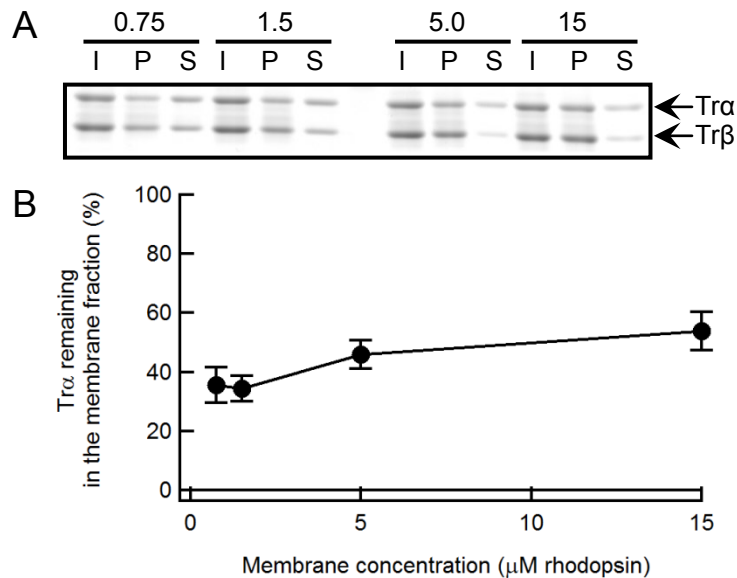


Figure 10. Membrane concentration dependent elution of Tr* from rod membranes

(A) Quantification of Tr α subunit by SDS-PAGE before and after centrifugation at membrane concentration indicated (μ M rhodopsin). Rod membranes were completely bleached in the presence of excess amount of GTP γ S. Membrane fractions were separated by centrifugation, and the supernatant and membrane fractions were treated with TCA. After heat treatment, samples were applied to 10 % acrylamide gel and electrophoresed and the gel was stained with CBB R-250. Total protein content was the same in each membrane concentration. I, the sample before centrifugation; P, membrane fraction after centrifugation; S, supernatant after centrifugation. Membrane fractions contained slight amounts of supernatant due to incomplete removal of the supernatant. (B) The remaining Tr α subunit in the membrane fraction. The horizontal axis expressed the concentration of rhodopsin as a membrane concentration. Each data point indicates mean \pm S.E. ($n = 3$).

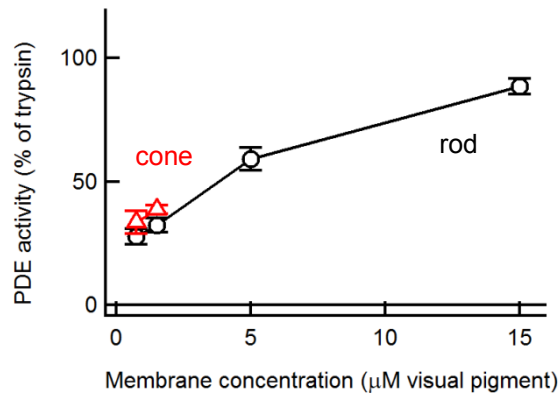


Figure 11. Membrane concentration dependent reduction of the full PDE activity in rod and cone membranes

PDE activities were measured by the pH assay method at indicated membrane concentrations by giving a light flash bleaching ~10 % of the visual pigment with excess amount of GTP γ S in rod and cone membranes. PDE activities were also measured by adding optimum concentrations of trypsin to activate all of PDE in a sample. PDE activities elicited by a light flash relative to those obtained in the trypsin-treated membranes were plotted against the membrane concentrations in rods (*circles*) and cones (*triangles*). Each data point indicates mean \pm S.E. ($n = 3$).

activity) and the activity of PDE treated with trypsin (trypsin-activated PDE activity) as a measure of the total activity of PDE in the sample (Fig. 11). At low visual pigment concentrations (0.75-1.5 μ M), the light-induced PDE activity was 30-40 % of the trypsin-activated PDE activity in both rod and cone membranes. The reduction of the activity was comparable with the portion of Tr* remaining in the membranes shown in Figure 10. In rod membranes, at high visual pigment concentration (15 μ M), the light-induced PDE activity was close (86 %) to the trypsin-activated PDE activity. However, at the same 15 μ M pigment concentration, only about half (53 %), which was slightly higher than the value at 0.75-1.5 μ M visual pigment (35 %), of the Tr* remained in the membranes (Fig. 10). Therefore, there seems to be a non-linear correlation between the portion of Tr* remaining in the membranes and the light-induced PDE activity. Because the reduction in the light-induced PDE activity was similar in rod and cone membranes at 0.75-1.5 μ M visual pigment, the concentration I used in this study, the extent of Tr* elution would have been similar between rod and cone membranes.

Reduction in the Dark PDE Activity in Cone Membranes in the Presence of GTP or GTP γ S

To measure the PDE activity in the presence of GTP or GTP γ S, firstly I tried to repeat the previous experiment where 0.5 mM GTP was present (Tachibanaki et al., 2001). However, in the case of cone membranes, it was difficult to measure the PDE activity, because the PDE activity in the dark was significantly high even at the starting point of the measurement (>50 % of the full PDE activity). Therefore, I modified the experimental conditions.

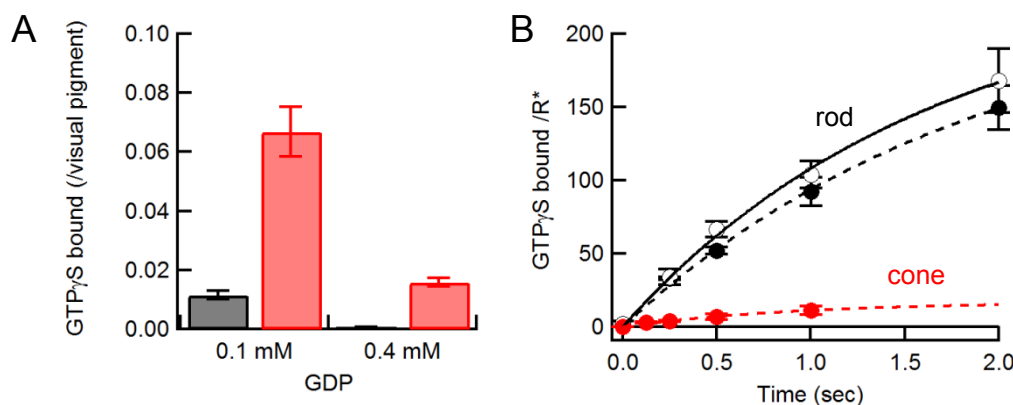


Figure 12. Time courses of Tr activation in rod and cone membranes

(A) GTPγS bound in the dark in rod and cone membranes. Each sample was mixed with GTPγS and incubated for 20 sec in rod (*black bars*, 3 μM rhodopsin) and cone (*red bars*, 1 μM visual pigment) membranes at 20°C. The binding is expressed as the number of GTPγS molecules bound per visual pigment present. (B) Light-dependent GTPγS-binding in rod and cone membranes. A light flash bleaching 0.027 % (rod) or 0.17 % (cone) of the visual pigment was given at time 0. The data points were fitted using an exponential curve ($Y=A/k \times [1-\exp\{-k \times t\}]$). The best fitted A and k values were, respectively, 145 GTPγS bound/ $R^* \cdot \text{sec}$ and 0.61 sec^{-1} in the presence of 0.1 mM GDP (*black solid curve*) and 121 GTPγS bound/ $R^* \cdot \text{sec}$ and 0.53 sec^{-1} in the presence of 0.4 mM GDP (*black dashed curve*) in rod membranes, and 18 GTPγS bound/ $R^* \cdot \text{sec}$ and 1.0 sec^{-1} in cone membranes in the presence of 0.4 mM GDP (*red dashed curve*). Each data point indicates mean \pm S.E. ($n = 2-3$).

The reason for the increase in PDE activity in the dark was probably because Tr was activated in the dark to some extent. Indeed, GTPγS-binding in the dark was much higher in cone membranes than in rod membranes in the presence of 0.1 mM GDP (Fig. 12A). To decrease the Tr activation in the dark, I increased GDP concentration to 0.4 mM. Under this condition, GTPγS-binding in the dark decreased to 25 % in cone membranes (Fig. 12A). I checked whether this increase in the GDP concentration affects the Tr activation rate (Fig. 12B). By fitting the time course of GTPγS binding to Tr and therefore that of Tr activation with an exponential curve, the rate of Tr activation was decreased only by ~17 % in rod membranes (from 145 GTPγS bound/ $R^* \cdot \text{sec}$, i.e. 145 $\text{Tr}^*/R^* \cdot \text{sec}$, to 121 $\text{Tr}^*/R^* \cdot \text{sec}$). Although the GTPγS-binding was not measured at 0.1 mM GDP in cone membranes in my study, the rate of Tr activation measured at 0.4 mM GDP (18 $\text{Tr}^*/R^* \cdot \text{sec}$) was close to the rate in the presence of 0.1 mM GDP measured in the previous study (30 $\text{Tr}^*/R^* \cdot \text{sec}$, Tachibanaki et al., 2012). The difference of the rate of Tr activation between rod and cone membranes was 5 (145/30) at 0.1 mM GDP and 7 (121/18) at 0.4 mM GDP. From this result, I concluded that although GDP reduces the Tr activation rate, GDP affects Tr activation almost equally in rod and cone membranes irrespective of its concentrations within the range I used.

High Rate of Hydrolysis of Nucleotides in Cone Membranes

I examined the hydrolysis of nucleotides in cone membranes during a measurement, because purified cones retain large ellipsoid regions (Figs. 1A and 3C) that probably contain the hydrolytic enzyme(s) for ATP and GTP. These nucleotides are essential for the reactions

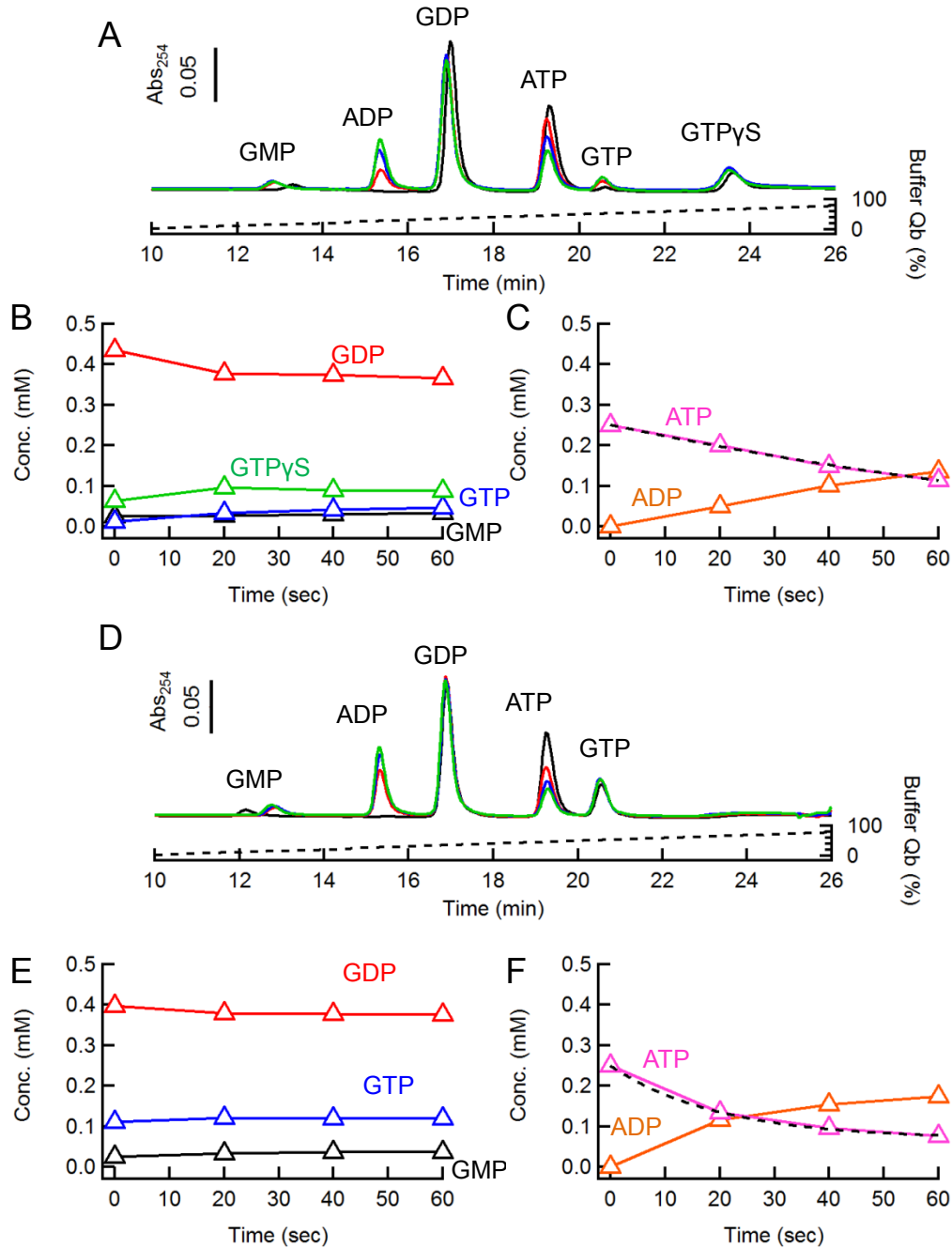


Figure 13. ATP hydrolysis in cone membranes

(A) ATP hydrolysis in the presence of GTPγS. Cone membranes containing 0.75 μM visual pigment were incubated with 0.25 mM ATP, 0.4 mM GDP and 0.1 mM GTPγS, and the reaction was quenched by addition of TCA at various time points (0 sec, *black trace*; 20 sec, *red trace*; 40 sec, *blue trace*; 60 sec, *green trace*). After centrifugation (20,400 × *g*, 15 min), the supernatant was loaded onto a Mono Q column and the nucleotides were analyzed. Concentration of each guanine nucleotide was determined from each peak area of the chromatogram by taking the sum of the areas being the total guanine nucleotide concentration (0.5 mM). Concentrations of ATP and ADP were determined similarly. (B and C) Time courses of the change in each nucleotide concentration (guanine nucleotides in B and adenine nucleotides in C) extracted from (A). (D) Similar as in (A), but in the presence of 0.25 mM ATP, 0.4 mM GDP and 0.1 mM GTP. Traces are the same as in (A). (E and F) Time courses of the change in each nucleotide concentration (guanine nucleotides in E and adenine nucleotides in F) extracted from (D). In (C) and (F), each time course of ATP hydrolysis was fitted using an exponential curve ($Y=A/B/[k \times [1-\exp\{-k \times t\}]]$). The best fitted *A*, *B* and *k* values were, respectively, 0.25 mM, 0.0029 mM/sec and 0.008 sec⁻¹ in (C) and 0.25 mM, 0.0090 mM/sec and 0.050 sec⁻¹ in (F) (*dashed curves*).

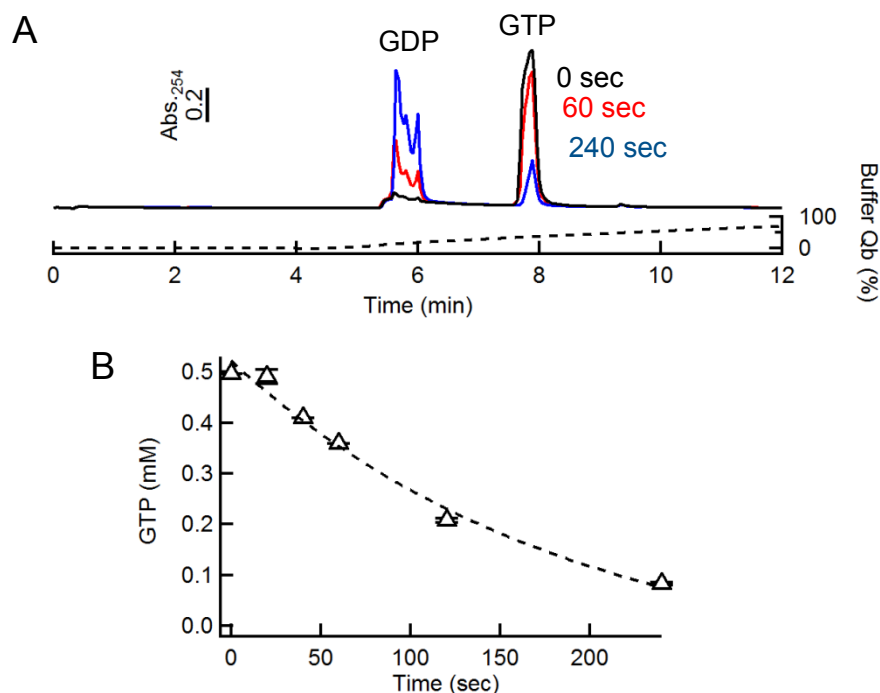


Figure 14. GTP hydrolysis in cone membranes in the absence of ATP

(A) Quantification of guanine nucleotides with Mini Q column chromatography. Cone membranes containing $0.75\ \mu\text{M}$ visual pigment were mixed with GTP, and the reaction was quenched by addition of TCA at indicated times (0 sec, *black trace*, 60 sec, *red trace*; 240 sec, *blue trace*). After centrifugation ($20,400 \times g$, 15 min), the supernatants were loaded onto a Mini Q column and nucleotides were detected by the absorbance at 254 nm. At the elution time of GDP, multiple peaks were observed probably because TCA was eluted at the same time. When the sample did not contain TCA, only a single peak was observed. Concentration of GTP was determined from the area of the elution peak of GTP. The area of the elution peak of GTP at 0 sec incubation was used as the initial concentration of GTP (0.5 mM). (B) The time course of GTP hydrolysis in cone membranes. The data points were fitted using an exponential curve ($Y=A-B/k \times [1-\exp\{-k \times t\}]$). The best fitted A , B and k values were, respectively, 0.52 mM, 0.0033 mM/sec and $0.0053\ \text{sec}^{-1}$ (*dashed curve*). Error bars show the deviation from the mean ($n = 2$).

in the phototransduction cascade, and are added in my PDE activity measurement. If these nucleotides are hydrolyzed rapidly, interpretation of my data will be complicated. The concentration of a nucleotide was determined by HPLC. As I expected, ATP hydrolysis was significant in cone membranes in the presence of GTP or GTP γ S (Fig. 13). By fitting the hydrolysis of ATP with an exponential curve, the initial rate of ATP hydrolysis was determined: it was $2.9\ \mu\text{M}/\text{sec}$ in the presence of GTP γ S and $9.0\ \mu\text{M}/\text{sec}$ in the presence of GTP (Fig. 13F) in the cone sample containing $0.75\ \mu\text{M}$ visual pigment. In Figure 13B, in the presence of GTP γ S, GTP concentration increased gradually: it was probably because GTP was produced from GDP by transfer of a high energy phosphate from ATP.

I also examined the changes in the nucleotide concentration in cone membranes during a measurement in the absence of ATP and found that GTP was significantly hydrolyzed in cone membranes in the absence of ATP (Fig. 14). The initial rate of GTP hydrolysis was $3.3\ \mu\text{M}/\text{sec}$ (Fig. 14B). Thus, nucleotide concentrations changed significantly in cone membranes during my measurements in cone membranes. Accordingly, I tried to complete each measurement as quickly as possible to minimize the changes in the concentration of

nucleotides: in cone membranes, nucleotides except cGMP were added 10-15 sec before giving a light flash. In rod membranes, nucleotide concentrations did not change significantly so that at 30 sec before bleaching, nucleotides were mixed. In the preliminary experiment, nucleotide concentrations did not change significantly in purified cone outer segment membranes. These results suggest that the ellipsoid region in the inner segment contains substantial amounts of the hydrolytic enzymes for ATP and GTP compared with the outer segment.