Cone photoreceptors show lower light sensitivity and briefer light responses than rod photoreceptors. The light detection signal in these cells is amplified through a phototransduction cascade. The first step of amplification in the cascade is the activation of a GTP-binding protein, transducin (Tr), by light-activated visual pigment (R*). We quantified transducin activation by measuring the binding of GTP-γS in purified carp rod and cone membrane preparations with the use of a rapid quench apparatus and found that transducin activation by an R* molecule is ~5 times less efficient in cones than in rods. Transducin activation terminated in less than 1 second in cones, more quickly than in rods. The rate of GTP hydrolysis in Tr*, and thus the rate of Tr* inactivation, was ~25 times higher in cones than in rods. This faster inactivation of Tr* ensures briefer light responses in cones. The expression level of RGS9 was found to be ~20 times higher in cones than in rods, which explains higher GTP hydrolytic activity and, thus, faster Tr* inactivation in cones than in rods. Although carp rods and cones express rod- or cone-version of visual pigment and transducin, these molecules themselves do not seem to induce the differences significantly in the transducin activation and Tr* inactivation in rods and cones. Instead, the differences seem to be brought about in a rod or cone cell-type specific manner.

In the vertebrate retina, there are two types of visual photoreceptors, rods and cones. They are different in many aspects. For example, the light sensitivity of a cone is lower than that of a rod, and the duration of a light response to a brief light flash is shorter in cones than in rods (for reviews, see Refs. 1–3). Because of the difference in light sensitivity, rods mediate night vision, whereas cones mediate daylight vision. Briefer light responses in cones enable them to detect light signals with higher time resolution than rods. These differences are thought to arise from the differences in the molecular mechanisms that are responsible for generation and termination of a light response. These mechanisms are well studied in rods. The generation mechanism, the phototransduction cascade, consists of visual pigment (R),2 photoreceptor-specific trimeric G-protein (transducin, Tr), and cGMP phosphodiesterase (PDE). A light-activated visual pigment molecule (R*) activates many molecules of Tr to amplify a photon capture signal by catalyzing the GTP-GDP exchange in the a subunit of Tr, and activated transducin (Tr*) then activates PDE. Activated PDE (PDE*) hydrolyzes cGMP to a GMP-gated cation channel to result in a hyperpolarizing light response. To terminate a light response, both inactivation of activated molecules (R*, Tr*, PDE*) and restoration of cGMP concentration are required. R* is inactivated by phosphorylation and subsequent binding of arrestin. Tr* is inactivated by hydrolysis of bound GTP to GDP with its intrinsic GTPase activity. It is known that RGS9, a photoreceptor-specific GTPase-accelerating protein (GAP), accelerates this hydrolysis (4).

In cones, homologous mechanisms are known to be present (1–3). In our previous studies, by using purified rods and cones from carp (Cyprinus carpio), we showed that PDE activation measured at its peak in the light is ~260 times less sensitive to light in cones than in rods (5), and R* inactivation by phosphorylation (6) together with restoration of cGMP concentration (7) is much more efficient in cones than in rods. These results seem to explain lower light sensitivity and briefer light responses in cones (3).

In this study, we focused on activation and inactivation of transducin in carp cones. Previously, we showed that transducin activation is less efficient in cones than in rods in carp (5). However, in that study, transducin activation by R* measured by binding of GTPγS, a nonhydrolyzable GTP analog, was completed in a few seconds in cones and was too rapid to measure with a conventional biochemical method. In this study, there-
fore, we used a rapid-quench apparatus to measure this time course and found that the rate of transducin activation is clearly lower in cones than in rods. We further measured the inactivation of transducin, i.e. the hydrolysis of GTP, and found that this is much faster in cones. It is very likely that this faster inactivation is due to an ~20 times higher expression level of RGS9 in the outer segment (OS) in cones.

**EXPERIMENTAL PROCEDURES**

**Preparation of Rod and Cone Membranes**—Animals were cared for according to the institutional guidelines. Carp (*C. carpio*) rods and cones were isolated by using a stepwise Percoll density gradient as described previously (5). Our cone preparations are a mixture of mainly red (~60%), green (~20%), and blue (~20%) cones (5). Isolated cells were washed with a potassium gluconate buffer (K-gluc buffer) (115 mM potassium gluconate, 2.5 mM KCl, 2 mM MgCl2, 10 mM HEPES, 1 mM DTT (pH 7.5)) and were stored at ~80 °C until use. When used, stored cells were thawed and washed twice by centrifugation with the K-gluc buffer to obtain rod or cone membranes, and then the membranes were suspended in the same buffer (5). We assumed that the access of small molecules like GTPγS, GTP, and ATP to membranes was not rate-limiting in our measurement. When necessary, we prepared membrane preparations containing intact visual pigments but lacking activities of peripheral proteins such as transducin and pigment kinase (6). Cone outer segment (COS)-rich and cone inner segment (CIS)-rich membranes were prepared as described previously (8). In each type of the sample, an aliquot of a suspension was used to quantify spectrophotometrically the amount of visual pigment in the membranes (5). All of these manipulations were performed in complete darkness with the aid of an infrared image converter (NVR 2015, NEC, Tokyo, Japan).

**Purification of Rod Transducin**—Rod transducin was prepared from purified rod membranes on the basis of a method reported in a previous report (9), with some modifications. Rod membranes were illuminated with a 100-W halogen lamp at a 20-cm distance to form a complex of R* and transducin. The membranes were washed once with a hypotonic buffer (5 mM Tris, 0.5 mM MgCl2, 1 mM DTT (pH 7.2)) supplemented with 0.2 mM EDTA and subsequently washed twice with the hypotonic buffer without EDTA to remove soluble and peripheral proteins, except transducin in the complex. Then, transducin bound to R* was extracted from rod membranes by using the hypotonic buffer supplemented with 1 mM GTP. The extract was concentrated in the K-gluc buffer with ultrafiltration (Vivaspin 20, Sartorius). Purified rod transducin was dissolved in an SDS-PAGE sample buffer (62.5 mM Tris, 10% (w/v) glyc- erol, 5% (w/v) β-mercaptoethanol, 2.3% (w/v) SDS, 0.1% (w/v) bromphenol blue) and was quantified with Coomassie Brilliant Blue (CBB)-staining after SDS-PAGE using BSA as a molar standard.

**GTPγS Binding Assay**—To measure time courses of transducin activation, a filter binding assay was carried out on the basis of a previous method using [35S]GTPγS (5). Rod or cone membranes (9 μl) were mixed with 6 μl of the K-gluc buffer containing various chemicals. The ingredients in the mixture were 0.6 μM rod or 0.3 μM cone pigments, 100 μM [35S]GTPγS, 100 μM GDP, 0.8 mM EGTA, and, when necessary, 1 mM ATP or 0.6 μM purified rod transducin. The concentration of GTPγS (100 μM) was saturating because 200 μM GTPγS gave results indistinguishable from those obtained at 100 μM GTPγS. After preincubation for 20 s, the sample was illuminated with a light flash at an attenuated intensity with use of a cutoff filter passing the light longer than 420 nm. In the experiments to measure time courses of transducin activation, light flashes bleaching 0.0085% and 0.25% of visual pigment in the rod and cone membranes, respectively, were used. These flash intensities were estimated on the basis of the neutral density filters we used for attenuation of a light flash of which intensity was calibrated separately. The measurement was carried out at 20 °C throughout. The binding of [35S]GTPγS was terminated at various time intervals after a light flash by adding 200 μl of an ice-chilled K-gluc buffer containing 50 mM NH4OH to inactivate R* plus 25 mM non-radioactive GTP to dilute radioactive [35S]GTPγS by >3000-fold. The timing of addition was strictly controlled by using a rapid-quench apparatus that could terminate a reaction in less than 0.1 s (6). In this study, we repeated measurements of a reaction at least three times independently, and all of the data points were pooled to determine the rate. In the measurement of GTPγS binding time course, the reaction started to become saturated at a few early time points, especially in the case of cone membranes (Fig. 2B). For this reason, we assumed a saturating exponential increase of GTPγS binding after a light flash and determined the initial rate of the binding of GTPγS with curve fitting.

**Quantification of Transducin**—The amount of transducin in rod and cone membranes was quantified using the GTPγS binding assay method described above, with some modifications. To activate all of the transducin molecules, a light flash bleaching 0.19 and 75% of visual pigments in rod and cone membranes, respectively, was given in the presence of 100 μM [35S]GTPγS. The reaction was terminated at 160 s and 5 s after the light flash in rod and cone membranes, respectively. To quantify the amount of transducin activated in the light, GTPγS binding in the dark was subtracted. The shorter incubation time in cone membranes was necessary to minimize light-independent thermal activation of transducin specifically observed in cones membranes.

**Measurement of GTP Hydrolysis by Tr**—In a single-turnover assay, GTP hydrolysis was measured as reported previously (10), with some modifications. A rod or a cone membrane suspension (10 μl) containing 3.34 mM adenosine 5'-[(β, γ-imido)-triophosphate (AMP-PNP) and 18.7 mM MgCl2 was illuminated with a light flash bleaching 75% (rod) and 95% (cone) pigment. After incubation for 40 s, the sample was mixed with 6.7 μl of the K-gluc buffer containing various chemicals. The ingredients in the mixture were 3 μM rod or cone visual pigment, 130 nM [ γ-32P]GTP, 2 mM AMP-PNP, 0.8 mM EGTA, 12 mM MgCl2. The measurement was carried out at 20 °C, and the reaction was terminated at various time intervals by addition of 50 μl of 6% (w/v) perchloric acid. Then, 350 μl of a phosphate buffer (5 mM NaH2PO4 (pH 7.4)) containing 10% (w/v) activated charcoal was added and mixed vigorously for 1 min three times every 10 min. The sample was centrifuged at 12,000 × g for 15 min at 4 °C, and 20 μl of the supernatant was used to measure
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the radioactivity of the liberated inorganic phosphate (Pi). GTP hydrolysis was determined by subtracting the activity measured in the dark. When the measurement was done at a high concentration of rod pigment (50 \( \mu \text{m} \)), 0.5 \( \mu \text{m} \) [\( \gamma^{32}\text{P} \)]GTP (final concentration) was used.

GTP hydrolysis was also measured under steady conditions. In this measurement, rod or cone membranes (9 \( \mu \text{l} \)) containing AMP-PNP and MgCl\(_2\) were mixed with 6 \( \mu \text{l} \) of the K-gluc buffer containing [\( \gamma^{32}\text{P} \)]GTP and EGTA. The ingredients in the mixture were 5 \( \mu \text{m} \) rod visual pigment or 1 \( \mu \text{m} \) cone visual pigment, 0.2 mm [\( \gamma^{32}\text{P} \)]GTP, 2 mm AMP-PNP, 0.8 mm EGTA, 12 mm MgCl\(_2\). After pre-incubation for 20 s, the sample was illuminated with a light flash bleaching 75% (rod) and 95% (cone) pigment. GTP hydrolysis was measured similarly as shown above except that the reaction was initiated by illumination, not by addition of GTP, and terminated by adding a larger volume (200 \( \mu \text{l} \)) of 6% (w/v) perchloric acid, and a larger volume (800 \( \mu \text{l} \)) of the phosphate buffer containing activated charcoal was used.

Cloning and Expression of Carp RGS9—To obtain a molar standard of RGS9, carp RGS9 was cloned by using a carp retinal cDNA library (11). The coding region of carp RGS9 (AB180757) was inserted into the Ncol/XhoI sites in pET-16b (Novagen, Darmstadt, Germany). The recombinant plasmid was introduced into Escherichia coli BL21DE3 (Novagen). Expression of RGS9 was carried out according to the instructions of the manufacturer. After the expression, cells were suspended in a Tris-buffered saline (100 mm Tris, 0.9% (w/v) NaCl (pH 7.4)), sonicated, and centrifuged (20,000 \( \times \) g for 10 min). RGS9 was collected in the insoluble fraction and was dissolved in an SDS-PAGE sample buffer. The amount of expressed RGS9 was quantified with Coomassie Brilliant Blue using BSA as a molar standard.

Preparation of Polyclonal Antisera against RGS9 and Quantification of RGS9 by Immunoblot Analysis—Whole coding region of carp RGS9 was inserted into the BamHI/XhoI sites of pGEX-5X-1 (GE Healthcare) and a GST-RGS9 fusion protein was expressed in E. coli BL21DE3. The fusion protein was purified according to the instructions of the manufacturer and was used to raise anti-RGS9 antiserum in mice. The antiserum was preadsorbed by a supernatant of lysed E. coli expressing GST to reduce nonspecific signals. The amount of RGS9 in rod or cone membranes was quantified by immunoblot analysis using a calibrated amount of RGS9.

RESULTS

Time Courses of Transducin Activation in Rod and Cone Membranes—In the measurement of transducin activation, we gave a light flash of a fixed intensity within a range where GTP\( \gamma \)S binding is a linear function of the flash intensity (0.0085% bleach in rod and 0.25% bleach in cone membranes, Fig. 1). Fig. 2, A and B, shows the time courses of transducin activation measured with binding of GTP\( \gamma \)S in the membrane preparations of carp rods (A) and cones (B). Activation was measured in the absence (black symbols) and presence (red symbols) of ATP. Note the large differences in the scales of x axes and y axes between Fig. 2, A and B. In these measurements, we used a rapid quench apparatus to terminate the reaction in a subsecond range (see "Experimental Procedures"). The binding is shown as the number of GTP\( \gamma \)S molecules bound per visual pigment.

![FIGURE 1. Transducin activation at various flash intensities.](image1.png)

![FIGURE 2. Early time courses of transducin activation in rod and cone membranes.](image2.png)

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pigment activated (R*). In this measurement, we gave a light flash to bleach only a small portion of visual pigment (0.0085% in rod and 0.25% in cone membranes) so that Tr molecules were present in excess over R* molecules. On the basis of the finding that the molar ratio of Tr to R is 0.094 in rods and 0.055 in cones (see Fig. 4A), Tr/R* ratios were > 1000 in rod and > 20 in cone membranes. In cone membranes, a higher intensity of light was necessary to obtain reliable binding signals of GTPγS.

In the absence of ATP and, therefore, in the absence of pigment phosphorylation, GTPγS binding time course was fitted with a simple exponential function (see “Experimental Procedures”) of:

$$Y = A[1 - \exp(-kt)]$$

where Y is the amount of GTPγS bound to transducin per R* (GTPγS/R*), A is the maximum amount of GTPγS bound to the transducin per R*, k is a rate constant, and t is the time after a light flash. Curve fitting of the results in rod membranes (Fig. 2A, black circle and dotted black line) gave the results of A = 497 GTPγS/R* and k = 0.29 s⁻¹. The initial rate of the binding obtained by the fitting, i.e., Ak, was 143 transducin activated per R* per sec (143 Tr*/R*-sec). A similar determination was made for the results in cone membranes (Fig. 2B, black circle and dotted black line). In cone membranes, A = 8.5 GTPγS/R* and k = 3.5 s⁻¹ and Ak = 30 Tr*/R*-sec. The time constants (1/k), 3.4 s in rod membranes (1/0.29), and 0.29 s in cone membranes (1/3.5), did not seem to be consistent with the decay time constant of meta II intermediate that has been believed to be a molecular species responsible for activation of transducin. They are more than 10 times higher and are generally several minutes in rods and several seconds in cones in intact cells (12, 13). No matter what k represents realistically in the GTPγS binding reaction, the above consideration gave us the estimation of the initial rate of GTPγS binding. 143 Tr*/R*-sec in rod membranes and 30 Tr*/R*-sec in cone membranes. The results showed that transducin activation was about 5 times (143/30) less efficient in cones. It was evident from this measurement that a significant portion of the difference in the amplification of a light detection signal in rods and cones is present at the level of transducin activation.

We further analyzed the GTPγS binding time course under the condition that the concentration of GTPγS is saturating (see “Experimental Procedures”). The reaction could be assumed to be

$$R^* + Tr \rightarrow Tr^*$$

REACTION 1

With k₁ as the rate constant of the binding of T to R*, k₂ as the decay rate constant of R*, [R]₀ and [Tr]₀ as the initial concentrations of R* and Tr, respectively, and t as the time after a light flash, we obtained a following equation that expresses a time course of GTPγS binding per R*:

$$
\frac{[Tr^*]/[R^*]_0 - ([Tr]_0/[R^*]_0)[1 - \exp(-k_1[R^*]_0/k_2)]}
\exp((k_1[R^*]_0/k_2)\exp(-k_2t))
$$

Curve fitting with Equation 2 gave results very similar to those obtained with Equation 1. The initial rates obtained with use of Equation 2 were 150 Tr*/R*-sec in rod membranes and 33 Tr*/R*-sec in cone membranes. In rod and cone membranes, respectively, the rate constants were k₁, 2.7 × 10⁶ M⁻¹·sec⁻¹ and 2.0 × 10⁶ M⁻¹·sec⁻¹, and k₂, 0.29 s⁻¹ and 3.5 s⁻¹.

In the presence of ATP (red circle and red dotted line in Fig. 2, A and B), the steady level of transducin activation was suppressed in both rod and cone membranes (compare black and red circle and red dotted line in Fig. 2A, A, and B). In cone membranes, the suppression was remarkably fast and already completed at our earliest time point (0.125 s). Although we could not determine the initial rate in the presence of ATP in cone membranes accurately, from the early data points in both rod and cone membranes it seems that ATP, and thus pigment phosphorylation, does not affect the initial rate of transducin activation. Instead, ATP or pigment phosphorylation facilitated termination of transducin activation in both rod and cone membranes. If R* activates transducin without significant delays, termination of transducin activation in the presence of ATP reflects inactivation of R* by phosphorylation. The result, therefore, suggests that R* inactivation is very rapid in cone cells.

**Rod Transducin Activation by Rod or Cone Visual Pigment**—In carp rods, rod versions of visual pigment and transducin, and in cones, their cone versions, are expressed (3). As shown in Fig. 2A and B, transducin activation was less efficient in cones than in rods. This difference is due to the difference between the activation of rod transducin by rod pigment in rods and the activation of cone transducin by cone pigment in cones. It was of interest whether rod transducin is activated in cone membranes at the efficiency similar to that in rod membranes or to that in cone membranes. To examine this, we measured GTPγS binding with addition of purified rod transducin to urea-washed (and thus endogenous transducin-free) rod and cone membranes in the absence of ATP (Fig. 2, C and D).

First, as a control, we added exogenous purified rod transducin to urea-washed rod membranes in an amount similar to that of endogenous rod transducin (added exogenous rod Tr/R ratio was 0.1, whereas endogenous rod Tr/R is 0.094, see Fig. 4A). The intensity of light flash was similar to that used in the measurement in Fig. 2A so that the ratio of Tr/R* was > 1000 in rod membranes. The red circle in Fig. 2C show GTPγS binding to this exogenously added rod transducin in urea-washed rod membranes. The initial rate of activation of exogenous rod transducin obtained by the fitting (108 Tr*/R*-sec, red dotted line) was lower (0.72 times) than but similar to that of endogenous rod transducin (143 Tr*/R*-sec, black dotted line, redrawn from Fig. 2A). The result indicated that exogenous rod transducin was activated at a similar rate as endogenous rod transducin. We then measured GTPγS binding in urea-washed and cone transducin-free cone membranes in the presence of exogenous rod transducin. In this measurement, rod transducin was added at the Tr/R ratio of 0.1 as in the control rod membranes. Because the endogenous cone Tr/R ratio is 0.055 (see Fig. 4A), the concentration of exogenous rod transducin was slightly higher (0.1/0.055 = 1.7) than that of endogenous cone transducin. The intensity of the light flash was similar to that used in Fig. 2B so that the total Tr/R* ratio was > 35. The added rod transducin
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FIGURE 3. Time courses of transducin inactivation in rod and cone membranes measured with a single-turnover assay method. A, light-dependent GTP hydrolysis was measured in rod (○, n = 3) and cone (∆, n = 3) membranes containing 3 μM pigment. The amount of released Pi was normalized to its maximum, and the data points were fitted by a single exponential function. Half-lives determined by the fitting were 47 and 3.0 s in rods and cones, respectively. B, light-dependent GTP hydrolysis was measured in rod membranes (○, n = 3) containing 50 μM pigment. The results are shown as in A. The rate of GTP hydrolysis was 0.074 Pi/Tr*-sec, and the half-life was 9.4 s. In A and B, each data point indicates mean ± S.E. in three independent measurements.

was activated by cone R* (Fig. 2D, red △), and the initial rate of GTPγS binding obtained by the fitting with a saturating exponential function (red dotted line) was 5.3 Tr*/R*-sec, which was 20 times (108/5.3) lower than the rate measured in the control rod membranes shown in Fig. 2C. It was evident that rod transducin is activated in cone membranes less efficiently than in rod membranes. Because of the difficulty in preparing large amount of cone transducin, measurement of activation using purified cone transducin was not possible.

Time Courses of Transducin Inactivation in Rod and Cone Membranes—So far, it has been speculated that inactivation of Tr* is more rapid in cones than in rods on the basis of the fact that the light response is much briefer in cones. To examine how rapidly Tr* is inactivated, we measured light-dependent GTP hydrolysis in cone membranes and compared it with that in rod membranes.

We first tried to measure the GTP hydrolysis with a single-turnover assay method (10). At a pigment concentration of 3 μM, the half-lives of Tr* in rod and cone membranes were 47 s and 3.0 s, respectively (Fig. 3A). Although the hydrolysis of GTP was faster in cone membranes than in rod membranes, the hydrolysis was too slow to explain the recovery time of a light response in both rods and cones. A typical subsaturated carp rod response terminates in 1 s, and a carp red cone response in 0.2 s (3). In a single-turnover assay, the GTP concentration should be lower than the transducin concentration. At 3 μM pigment concentration, Tr concentration was expected to be 0.3 μM or so. For this reason, GTP concentration we used was 0.13 μM, and it probably was too low for immediate diffusional binding of GTP to the GTP-free form of transducin (10). In fact, we observed more rapid GTP hydrolysis (half-life, 9.4 s, Fig. 3B) in rod membranes at a higher concentration of pigment (50 μM) and GTP concentration (0.5 μM). However, preparation of cone membranes of high pigment concentration was practically very difficult because of limited availability of cone photoreceptors. For this reason, instead of use of single-turnover assay methods, we measured hydrolysis of GTP under a steady condition. We gave an intense light flash to activate all of transducin molecules in the presence of saturating concentrations of GTP to measure the maximal rate of GTP hydrolysis.

To measure how many GTP molecules are hydrolyzed per Tr* molecule, we needed to quantify the amount of transducin in the OS of rods and cones. By giving an intense light flash to activate all of the transducin molecules, the maximum amount of the binding of GTPγS in the rod and cone membranes was determined. Because our purified rods and cones were obtained under complete darkness, rod transducin should have been localized only to the OS. The maximum binding was 0.094 ± 0.003 (mean ± S.E., n = 3) molecules of GTPγS bound per visual pigment present in rod membranes and 0.055 ± 0.004 in cone membranes (n = 3) (Fig. 4A). The result indicated that cone transducin is 0.6 times more abundant in the cone OS than
rod transducin in the rod OS. On the assumption that the pigment concentration is 3 mM in both rods and cones (14), transducin concentration in the OS was calculated to be 0.28 mM in rods and 0.17 mM in cones.

Fig. 4B shows the amounts of released Pi per transducin present in rod (●) and cone (▲) membranes. From these measurements, initial rates of GTP hydrolysis were determined in rod and cone membranes (straight lines). Because all of the transducin molecules should have been activated at the initial phase of the reaction, the initial rate of hydrolysis per Tr* could be calculated. The rate in cone membranes was 2.1 Pi released per Tr* per sec (2.1 Pi/Tr*-sec) and was ~25 times higher than that in rod membranes (0.082 Pi/Tr*-sec). The half-lives of Tr* calculated from the rates of hydrolysis are 8.5 s in rod membranes and 0.33 s in cone membranes. Our results clearly indicated faster Tr* inactivation in cones than in rods.

Inactivation of Exogenous rod Tr* in Rod and Cone Membranes—As shown in Fig. 2, rod transducin was activated 20 times less efficiently in cones than in rods. Then, it was of interest whether activated rod transducin is inactivated in cones at a similar rate as that of cone transducin in cones or as that of rod transducin in rods. To examine it, firstly as a control, GTP hydrolysis was measured in rod membranes in the presence of both endogenous rod transducin and exogenously added rod transducin (the ratio of exogenous rod transducin to endogenous rod transducin was ~2:1). We did not use urea-washed membranes in this study because activities of many of outer segment proteins, including a GAP protein, RGS9, would be affected by this wash (15). The intensity of a light flash was similar to that used in Fig. 4B so that all of the transducin molecules should have been activated. The initial rate of GTP hydrolysis per transducin present (and thus per Tr*) was 0.057 Pi/Tr*-sec (red ● and red straight line in Fig. 4C). Note that the rate was calculated in a unit of per Tr* so that the hydrolysis by both endogenous and exogenous rod transducin was averaged. The rate was 70% of that measured in the presence of only endogenous rod transducin in native membranes (0.082 Pi/Tr*-sec, black straight line in Fig. 4C, redrawn from B). This less efficient GTP hydrolysis (Fig. 4C) could be explained by reduced population of a complex of Tr* and RGS9 relative to the total number of transducin molecules (see “Discussion”).

In cone membranes, a similar measurement was made in the presence of 3.6 times the excess amount of exogenous rod transducin over endogenous cone transducin. The averaged rate in the presence of exogenous rod transducin was 0.96 Pi/Tr*-sec (Fig. 4D, red ▲ and red straight line). This rate was about a half (46%) of the rate of endogenous cone Tr* (2.1 Pi/Tr*-sec, black straight line in Fig. 4D) but 12 times higher (0.96/0.082) than the rate measured in the presence of endogenous rod Tr* in rod membranes. Assuming that exogenous rod transducin does not affect the GTP hydrolysis by endogenous cone transducin and that exogenous rod transducin hydrolyzes GTP at the same rate as that in native rod membranes (i.e. 0.082 Pi/Tr*-sec), our simple calculation showed that the averaged hydrolysis of GTP in the measurement in Fig. 4D should be lower than the measured activity (0.96 Pi/Tr*-sec) and was expected to be 0.52 Pi/Tr*-sec. In other words, to account for the measured activity, exogenous rod transducin should have hydrolyzed GTP at an ~8 times higher rate (0.64 Pi/Tr*-sec) than the rate of rod transducin in native rod membranes. In reality, some of RGS9 molecules in cone membranes would have bound to the exogenous rod Tr* instead of endogenous cone Tr* so that the GTP hydrolytic activity of exogenous rod transducin in cone membranes could have been increased more than 8 times. In summary, in the studies in both Fig. 2 and 4, it became evident that, to some extent, rod transducin is activated and inactivated like cone transducin in cone membranes. In cone membranes, transducin activation was 5 times less efficient, and its inactivation was 25 times more effective than in rod membranes, whereas rod transducin was activated 20 times less efficiently and inactivated at least 8 more less efficiently in cone membranes than in rod membranes.

Quantification of RGS9 in Rods and Cones—As shown in Fig. 4, exogenous rod transducin hydrolyzed GTP in cone membranes at a rate much higher than that in rod membranes. One of the possible mechanisms of this effect is the difference in the expression levels of RGS9 between rods and cones. Although the expression level of RGS9 has been reported to be higher in cones than in rods (15, 16), quantitative comparison between rods and cones in the same animal species has not been made. We compared it in our purified carp rods and cones.

So far, no cell-type specific RGR9 subtype has been reported, and in accordance with this notion, we isolated only one type of RGS9 mRNA in our carp retinal cDNA library (11). We therefore raised an antibody against recombinant carp RGS9 expressed in E. coli and performed an immunoblot analysis on the membrane fraction and the soluble fraction of purified rod and cones (Fig. 5A). In accordance with a previous study, RGS9 was found exclusively in the membrane fraction in rods (17) as well as in cones (Fig. 5A). An immunopositive signal was found at the same position on the SDS-PAGE in the rod and the cone sample (arrows), which supports the notion that rods and cones in carp express the same subtype of RGS9.

We then quantified the amount of RGS9 molecules expressed in rods and cones with use of recombinant RGS9 as a molar standard (Fig. 5B). The result showed that the molar ratio of RGS9 to visual pigment was 0.0011 ± 0.0004 (mean ± S.E., n = 5) in rods. This level was consistent with the RGS9/rhodopsin ratio of 0.006 – 0.0016 in mouse and bovine reported previously (4, 16). In cones, the ratio was 0.025 ± 0.0024 (n = 8), which is 1.6 times higher than the ratio estimated in cone-dominant chipmunk retina (16).

Distribution of RGS9 in Cones—It has been shown immuno-histochemically that RGS9 is mostly localized in the OS both in rods and cones (15, 16). However, their immunopositive signals were found in the cone inner segment (CIS) (15). Because our purified cones retain a portion of inner segment (IS) (for example, see Fig. 1A in Ref. 3), we tried to know the concentration of RGS9 in the OS. For this purpose, we prepared cone membrane samples having different OS/IS ratios (COS/CIS). The amounts of RGS9 were quantified in these samples and are expressed as the molar ratio to visual pigment present in the sample. The RGS9/pigment ratio thus determined was plotted as a function of the COS/CIS ratio (Fig. 5C). The result showed that the RGS9/pigment ratio did not depend on the COS/CIS ratio. Because visual pigment is present only in the OS, the constant
RGS9/pigment ratio irrespective of the COS/CIS ratio in Fig. 5C indicated that RGS9 in our isolated cones is almost exclusively distributed in the OS. This conclusion is consistent with a previous report (15) that RGS9 in the cone IS is distributed at the basal part of ellipsoid and at the synaptic terminal, both of which our purified cones are devoid of. Assuming that the pigment concentration is 3 mM (14), from our quantification in Fig. 5B, RGS9 concentration in the cone OS was calculated to be ~75 μM, which is > 20 times higher than the RGS9 concentration in the rod OS (3.3 μM). On the basis of the quantified amount of transducin in the OS in rods and cones (Fig. 4A), the molar ratio of transducin to RGS9 is 85:1 (0.28 mM/3.3 μM) in rods and 2.3:1 (0.17 mM/75 μM) in cones.

**DISCUSSION**

In this study, we showed that in cone membranes the rate of transducin activation was 5 times lower (Fig. 2, A and B); that transducin activation terminated very quickly (Fig. 4B); that GTP hydrolysis, and thus the inactivation of Trγ, was 25 times faster when all of the transducin molecules were activated (Fig. 4B); and that rod transducin behaves like cone transducin to some extent in cone membranes (Fig. 2, C and D, and Fig. 4, C and D). It was suggested that the faster GTP hydrolysis in cones is due to a > 20 times higher expression level of RGS9 in the cone OS (Fig. 5).

**Lower Efficiency of Transducin Activation in Cones**—In this study, the rate of transducin activation in cones was 30 Trγ/R*--sec (Fig. 2B) and was 5 times lower than that in rods (143 Trγ/R*--sec). In a recent study, it was found that the concentration of transducin is linearly related to the activation efficiency of transducin by R* (18). As shown in Fig. 4A, cone transducin is about 2 times less abundant in cones than in rods. When this difference in the expression level of transducin is taken into account, about a half of the 5 times lower rate of transducin activation in cones is due to the decreased level of transducin in cones.

In the presence of ATP, termination of transducin activation was greatly facilitated in both rods and cones (Fig. 2, A and B). The effect of ATP could be mainly attributed to visual pigment phosphorylation, a quench mechanism of R*. Generally, the rate of phosphorylation per R* decreases when more pigment is present in the presence of ATP, termination of transducin activation was 5 times lower (Fig. 2, A and B). The effect of ATP could be mainly attributed to visual pigment phosphorylation, a quench mechanism of R*. Generally, the rate of phosphorylation per R* decreases when more pigment is bleached (5). The bleach level was 30 times higher in the study in cone membranes than in rod membranes in this study (0.25% bleach in cones versus 0.0085% bleach in rods). Nonetheless, in the presence of ATP, termination of transducin activation was much faster in cones (< 0.125 s, Fig. 2B) than in rods (~ 10 s, A) after a light flash. This result shows that visual pigment phosphorylation is much more effective in cone membranes. This higher effect of ATP and, thus, very effective visual pigment phosphorylation in cone membranes is probably due to 100 times higher activity of visual pigment kinase in rods than in cones (6). This is the first biochemical demonstration of the effect of visual pigment phosphorylation on the termination of transducin activation in cones.

**Determiant of Less Efficient Transducin Activation in Carp Cones**—With mathematical analysis, Pugh and Lamb (19) formulated the amplification constant as an index of the degree of amplification in the activation step of phototransduction cas-
Transducin Activation and Inactivation in Cones

We tried to estimate the GTP hydrolytic activity of RGS9-free and RGS9-bound form of cone Tr*. However, the activity of RGS9-free form of rod Tr* estimated above was too low to explain the rate (0.96 Pi/Tr*-sec) measured in cone membranes in the presence of exogenous rod transducin. It seemed that in cone membranes, the RGS9-free form of rod Tr* shows a GTP hydrolytic activity higher than that in rod membranes. Because there were many unknown factors, such as the difference in the binding affinity of RGS9 between rod and cone transducin, further analysis was not made.

In this study, we showed that termination of transducin activation by visual pigment phosphorylation (Fig. 2B) and inactivation of transducin (Fig. 4B) are much faster in cones. In addition, we showed previously that cGMP synthesis is much more efficient in cones than in rods (7). All these very effective inactivation mechanisms contribute not only to shorten a light response but also to reduce the dark noise in cones even though visual pigment is spontaneously activated in these cells (24). These effective inactivation mechanisms, together with lower efficiency of transducin activation, seem to explain low light-sensitivity and a brief light response in cones.

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