Dephosphorylation during Bleach and Regeneration of Visual Pigment in Carp Rod and Cone Membranes*

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**Background**: Light-activated visual pigment is inactivated by phosphorylation and then regenerated. In this regeneration cycle, phosphates incorporated should be removed.

**Results**: Dephosphorylation was more effective in cones than in rods and did not show substrate preference during the cycle.

**Conclusion**: Phosphorylated visual pigment is dephosphorylated constantly in the regeneration cycle in both rods and cones.

**Significance**: A mechanism of visual pigment regeneration is known.

On absorption of light by vertebrate visual pigment, the chromophore, 11-cis retinal, is isomerized to all-trans retinal to activate the phototransduction cascade, which leads to a hyperpolarizing light response. Activated pigment is inactivated by phosphorylation on the protein moiety, opsin. Isomerized all-trans retinal is ultimately released from opsin, and the pigment is regenerated by binding to 11-cis retinal. In this pigment regeneration cycle, the phosphates incorporated should be removed in order that the pigment regains the capability of activating the phototransduction cascade. However, it is not clear yet how pigment dephosphorylation takes place in the regeneration cycle. First in this study, we tried to estimate the dephosphorylation activity in living carp rods and cones and found that the activity, which is present mainly in the cytoplasm in both rods and cones, is three times higher in cones than in rods. Second, we examined at which stage the dephosphorylation takes place; before or after the release of all-trans retinal, during pigment regeneration, or after pigment regeneration. For this purpose we prepared three types of phosphorylated substrates in purified carp rod and cone membranes: phosphorylated bleaching intermediate, phosphorylated opsin, and phosphorylated and regenerated pigment. We also examined the effect of pigment regeneration on the dephosphorylation. The results showed that the dephosphorylation does not show substrate preference in the regeneration cycle and suggested that the dephosphorylation takes place constantly. The results also suggest that, under bright light, some of the regenerated visual pigment remains phosphorylated to reduce the light sensitivity in cones.

In the vertebrate retina there are two types of photoreceptor cells, rods and cones. In both cells visual pigment (R), composed of a chromophore, 11-cis retinal, and a protein moiety, opsin, absorbs light to isomerize 11-cis retinal to all-trans retinal to induce a conformational change in opsin. Once all-trans retinal is formed, the pigment is activated to lead to activation of an enzymatic cascade known as the phototransduction cascade to evoke a hyperpolarizing light response (1, 2). Activated pigment is finally bleached through bleaching intermediates and is decomposed into all-trans retinal plus opsin so that the pigment should be regenerated for rods and cones to maintain their ability to detect light. Regeneration of the pigment is attained by binding of opsin to 11-cis retinal supplied with mechanisms known as the retinoid cycle or the visual cycle (3). In this way visual pigment is recycled (visual pigment regeneration cycle). In the phototransduction cascade, the activated pigment (R*) is inactivated by phosphorylation with G protein-coupled receptor kinases and/or binding of arrestin (4, 5). For the pigment to fully recover its ability to activate the phototransduction cascade, the phosphates incorporated into opsin should be removed.

It is well known that the sensitivity to light is higher in rods than in cones, and thus, rods mediate night vision, whereas cones mediate daylight vision. It is known that rods are easily saturated with weak light. However, cones are not saturated even under very bright light that can bleach potentially almost all cone pigment in <0.1 s (6), which strongly suggests that visual pigment having the capability of activating the phototransduction cascade is regenerated in cones very effectively. In fact, very effective supply of 11-cis retinal has been known to be present only in cones (7–10), but little is known about removal of the phosphates incorporated into opsin in rods or cones.

Removal of phosphates (dephosphorylation) from phosphorylated opsin has been shown in living animals (11–14). Although biochemical studies have been made previously (15, 16), it has not been known clearly at which stage in the regeneration cycle dephosphorylation takes place. Previous suggestions were that dephosphorylation takes place after decay of the light-activated protein (15) or after regeneration of visual pigment (13). Most of these studies were made in rods, and little is known about the dephosphorylation reaction in cones.
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Although it has been suggested that the dephosphorylation is faster in cones than in rods (14), comparison was made between zebrafish cones and mouse rods in that study. In the present study, therefore, we first compared the rate of dephosphorylation between rods and cones in the same animal species using membranes and cytoplasmic proteins of purified rods and cones obtained from carp retina. Second, to examine at which stage in the regeneration cycle dephosphorylation takes place most effectively, we prepared phosphorylated substrates at various stages and measured dephosphorylation activities in these preparations; they were phosphorylated bleaching intermediates produced after a light flash (BI-P), phosphorylated opsin (Opsin-P), and phosphorylated and regenerated visual pigment (VP-P), all in carp rod and cone membranes. Furthermore, we measured dephosphorylation activities in BI-P in the presence of 11-cis retinal to examine the effect of pigment regeneration.

Experimental Procedures

Preparation of Membranes and Cytoplasmic Proteins of Rods and Cones—Carp (Cyprinus carpio) rods and cones were purified as described previously (17). Animal care was conducted according to the institutional guidelines (approval number FBS-14-005). Purified cells were washed first with Ringer’s solution (119.9 mM NaCl, 2.6 mM KCl, 0.5 mM CaCl₂, 0.5 mM FBS-14-005). Purified cells were washed first with Ringer’s (molar ratio of the pigments, red:green:blue:UV 3:1:1:negligible) (18). The supernatant was combined with that obtained in the previous centrifugation, and the resultant cytoplasmic proteins were concentrated using Vivasin 20 (M, 10,000 cut-off, GE Healthcare). We confirmed that our cytoplasmic proteins contain ≥ 80% of soluble proteins that can be obtained with repetitive freeze-thaws. The amount of the cytoplasmic proteins was quantified by Bradford method using bovine serum albumin (BSA) as a molar standard. In some of the studies in cone membranes (Figs. 1, 2 and 5), we used very concentrated cytoplasmic proteins (>20 mg/ml). This was because the content of cytoplasmic proteins per single cells was much larger in cones than in rods (see “Results and Discussion”). In other studies (Figs. 3 and 4), we used cone cytoplasmic proteins at a low concentration (2 mg/ml), which was similar to that of rod cytoplasmic proteins used in the measurement in rod membranes. The effect of the cytoplasmic proteins varied slightly depending on the batches prepared.

Quantification of Visual Pigments in Rod and Cone Membranes—Concentrations of visual pigments in rod and cone membranes were determined spectrophotometrically. Carp rod and cone pigments contain A₂-type 11-cis retinal. To quantify the A₂-type visual pigment in carp, we obtained A₁-type rod pigment and A₂-type rod pigment, both regenerated from opsin in rod membranes. Assuming that the molar extinction coefficient at the absorption maximum (εₘₐₓ) of A₁-type rod pigment is 40,000 and that the same amounts of rod pigment are regenerate by the addition of an excess amount of A₁-type or A₂-type 11-cis retinal, we found that the εₘₐₓ of A₁-type rod pigment is 32,000, in agreement with the value reported previously (19). The εₘₐₓ of A₂-type red-sensitive cone pigment was assumed to be 38,400 based on a previous study that the εₘₐₓ of red-sensitive cone pigment is 1.2 times higher than that of rod pigment (20). Pigment concentrations in carp rod and cone membranes were determined based on these εₘₐₓ values.

Preparation of Photoreceptor Membranes Containing Opsin-P and VP-P—To prepare rod membranes containing Opsin-P or VP-P, rod membranes in K-gluc buffer containing 0.5 μM rhodopsin, 1 mM [γ⁻³²P]ATP, 0.5 mM GTP, and 0.6 mM EGTA in K-gluc buffer was exposed to yellow light (>410 nm) from a 150 watt tungsten/halogen lamp for 10 min at a distance of 8 cm at room temperature to phosphorylate bleaching intermediates of rhodopsin in the membranes. Then, to bleach rhodopsin fully and to remove isomerized all-trans retinal completely, the membranes were illuminated for another 10 min with the same illumination in the presence of 10 mM NH₂OH. We confirmed that rhodopsin was fully bleached with these illuminations. Then the membranes were washed once with K-gluc buffer containing 1% (w/v) BSA and 50 mM NH₂OH to remove unreacted [γ⁻³²P]ATP and all-trans retinal, with K-gluc buffer containing 1% BSA, and finally with K-gluc buffer twice, all by centrifugation at 100,000 x g for 10 min, and the membranes were resuspended in K-gluc buffer. These membranes containing phosphorylated- and all-trans retinal-free opsin were used as the source of Opsin-P and VP-P (see below).

To obtain the membranes containing VP-P, a 10 x molar excess of 11-cis retinal (A₁-type) dissolved in ethanol was added to the membranes containing phosphorylated- and all-trans retinal-free opsin. After the membranes were incubated for 30 min at 20 °C in the dark, the membranes were washed once by K-gluc buffer containing 1% BSA and 50 mM NH₂OH to remove excess 11-cis retinal and successively washed by K-gluc buffer containing 1% BSA once. The membranes were additionally washed twice with K-gluc buffer to replace the buffer solution and were used as the membranes containing VP-P. As a result, 102 ± 16% (n = 3, mean ± S.D. throughout this study) of rhodopsin was regenerated. We prepared rod membranes contain-
plation levels or of dephosphorylation: 2.8 ± 0.2 phosphates were present per Opsin-P (n = 3) and 2.2 ± 0.1 phosphates per VP-P (n = 12). The maximum level of the phosphorylation in our native rod membranes was three per R* (17), so that most of the phosphates incorporated into the pigment were retained in Opsin-P and VP-P in rod membranes.

Prepared Opsin-P and VP-P were used immediately after their preparation. It took ~3.5 h to prepare Opsin-P and VP-P in rod membranes, and the phosphorylation levels were similar in these preparations at the beginning of the measurement of phosphorylation levels or of dephosphorylation: 2.8 ± 0.2 phosphates were present per Opsin-P (n = 3) and 2.2 ± 0.1 phosphates per VP-P (n = 12). The maximum level of the phosphorylation in our native rod membranes was three per R* (17), so that most of the phosphates incorporated into the pigment were retained in Opsin-P and VP-P in rod membranes.

Cone membranes containing Opsin-P or VP-P were prepared similarly as rod membranes but with some modifications. To phosphorylate cone pigments, cone membranes were illuminated with yellow light (>410 nm) from the 150-watt tungsten/halogen lamp for 5 min at room temperature. To bleach cone pigments further and to remove isomerized all-trans retinal, the membranes were illuminated with the yellow light for another 1 min in the presence of 100 μM NH₂OH (NH₂OH concentration was reduced compared with that used for rod membranes because cone pigments are more sensitive to NH₂OH). The bleach level of the pigment was 94.5 ± 3.2% (n = 3). Regeneration level of the pigments by the addition of 11-cis retinal was 101 ± 13% (n = 3). It took ~2.5 h to prepare Opsin-P and VP-P in cone membranes, and the phosphorylation levels were similar in Opsin-P and VP-P: 2.5 ± 0.1 phosphates were present per Opsin-P (n = 3) and 2.5 ± 0.1 phosphates per VP-P (n = 12). Because the maximum phosphorylation level was three phosphates per R* in native cone membranes (17), most of the phosphates were retained in Opsin-P and VP-P also in cone membranes.

**Measurements of Phosphorylation Levels in BI-P, Opsin-P, and VP-P**—We measured dephosphorylation activities at three different stages of the pigment regeneration cycle: BI-P, Opsin-P, and VP-P. In the measurement of dephosphorylation activity in BI-P, time course of phosphorylation levels were measured in rod or cone membranes after a light flash. These membranes in K-gluc buffer containing 0.5 μM visual pigment, 1 mM [γ-32P]ATP, 0.5 mM GTP, and 0.6 mM EGTA were preincubated for 30 s in the dark and illuminated with a light flash bleaching 65% of rod pigment or 80% of cone pigments. After incubation for a desired time, a 20-μl aliquot was withdrawn and mixed with 100 μl of quenching buffer (200 mM KCl, 100 mM NaF, 5 mM adenosine, 200 mM EDTA in 250 mM sodium phosphate buffer, pH 7.2) based on a method reported previously (11). After centrifugation (20,000 × g for 15 min), the precipitate was washed twice with K-gluc buffer and suspended in SDS sample buffer (62.5 mM Tris-HCl, 2.3% (w/v) SDS, 10% (w/v) glycerol, 0.0025% bromphenol blue, 1.25% (w/v) 2-mercaptoethanol, pH 6.8). After SDS-PAGE, the amount of 32P present in the band of visual pigment was quantified using an image analyzer (BAS 2500, Fuji Film). In both rod and cone membranes, the amount of visual pigment was quantified by Coomassie Brilliant Blue staining after SDS-PAGE using BSA as a molar standard. The band of cone pigment in our SDS-PAGE was too broad and blurred in our Coomassie Brilliant Blue staining at the quantity of cone membranes in an aliquot withdrawn. In our purified cones, F₁ ATPase, which is a membrane-bound protein in the inner segment membranes, is abundantly present and could be a marker protein of the quantity of cone cells. For this reason the ratio of the amount of F₁ ATPase β subunit to that of cone pigment was determined each time when cone membranes were prepared, and the amount of cone pigments in an aliquot withdrawn was estimated from the amount of F₁ ATPase β subunit in the same aliquot with Coomassie Brilliant Blue staining after SDS-PAGE. When the effect of the cytoplasmic proteins was examined, the proteins were added to rod or cone membranes at the desired concentrations. Phosphorylation levels in Opsin-P and VP-P were measured similarly as in BI-P.

It should be mentioned that we measured phosphorylation levels in BI-P in the dark after a light flash. In this case, probably phosphorylation and dephosphorylation were both taking place because ATP was present during incubation and because R* should be present for a certain period of time after a light flash. Our attempt to wash out free [γ-32P]ATP by centrifugation to terminate phosphorylation and to measure only dephosphorylation was not effective, because the measurement of early time course of phosphorylation levels was not possible. In contrast, in the measurement of phosphorylation levels in Opsin-P and VP-P, only dephosphorylation was measured. This is because ATP was not added during incubation in these measurements. It has been shown that the dephosphorylation time course is dependent on how many phosphates are incorporated and from which phosphorylation sites a phosphate is removed (13), which means that the dephosphorylation time course is complex when the pigment is multiply phosphorylated. In our study we prepared BI-P, Opsin-P, and VP-P by bleaching visual pigment at least 65%, and under these conditions these substrates are all multiply phosphorylated. For this reason, dephosphorylation time course would be composed of multiple exponential functions (see below).

When the phosphorylation level in BI-P, Opsin-P, or VP-P decreased during incubation, this decrease was fitted to an exponential function to determine the rate of dephosphorylation. However, a single exponent did not give good fitting, which is probably because of the reason mentioned above. For a practical reason, therefore, we used a double exponential function as a measure of a dephosphorylation reaction.

\[
P(t) = P_1 \exp(-k_1 \times t) + P_2 \exp(-k_2 \times t) \tag{1}
\]

where \(t\) is the time after the start of the measurement, \(P(t)\) is the number of phosphates bound per R*(P/R*), per opsin (P/opsin), or per pigment present (P/R) at time \(t\), \(P_1\) and \(P_2\) are practical initial numbers of phosphates expressed in the unit of P/R*, P1/opsin, or P2/R in the fast component and the slow component, respectively, and \(k_1\) and \(k_2\) are the rate constants of the fast and the slow component, respectively. In the actual determination of the rate of dephosphorylation, we obtained the initial rate of dephosphorylation by calculating \(P_1k_1 + P_2k_2\) after fitting. In this study the rate of dephosphorylation shown is the initial rate of dephosphorylation obtained in this way. When the rates are compared under different conditions such as the
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measurements in the presence and absence of the cytoplasmic proteins, $P_1$ and $P_2$ were fitted so as to be the same under different conditions.

Dephosphorylation activities are slightly different among the batches of rod or cone membranes prepared. It could be because elution of phosphatase(s) in membranes differs in each preparation. As stated above, dephosphorylation activities induced by the cytoplasmic proteins, too, are slightly different in different batches. For these reasons we used same batches of membranes and cytoplasmic proteins in a single set of study.

Cytoplasmic proteins obtained in this study contain proteins that possibly affect phosphorylation and/or dephosphorylation of the pigment. Among them, S-modulin (or recoverin) could inhibit pigment phosphorylation. However, the Ca$^{2+}$ concentration in K-gluc buffer we used is low, so that inhibition by S-modulin would be negligible (21). Dephosphorylation of R* is inhibited by the GTP-free form of transducin, but it is not inhibited in the presence of GTPγS, an analogue of GTP (22).

To minimize the effect of transducin, therefore, we added GTP to rod or cone membranes when the effect of transducin is likely present. There is an additional reason that probably allows us to exclude the possibility of the contribution of transducin in the effect of the cytoplasmic proteins. First, transducin is present in our rod and cone membranes, not in the cytoplasmic proteins. It is because these membranes were prepared in the dark and in the absence of GTP. Second, a regulator of transducin, RGS9, is also present in the membranes (23). As we described above, transducin may minimally contribute to the inhibition of phosphorylation in our study, but its effect is always present no matter whether the cytoplasmic proteins are added or not. Therefore, the effect observed in the presence of the cytoplasmic proteins is surely caused by some proteins, but it is not by transducin. Arrestin is one of the proteins in the cytoplasmic proteins and could inhibit dephosphorylation (24) by binding firmly to phosphorylated pigment. This possibility is discussed under “Results and Discussion.”

Measurements of Phosphorylation Levels in the Presence of Pigment Regeneration in Rod and Cone Membranes—Dephosphorylation activities were also measured in the presence of pigment regeneration. Rod and cone membranes were illuminated by a light flash of >410-nm light similarly as in the dephosphorylation measurement in BI-P, but to examine the effect of pigment regeneration the study was made in the presence of 11-cis retinal. Under this condition, rod pigment in rod membranes or cone pigments in cone membranes were bleached, but 11-cis retinal is not isomerized to other isomers with this light. Then we could expect pigment regeneration in the dark after the light flash in both rod and cone membranes. Dephosphorylation levels were measures as in BI-P.

Measurement of Pigment Regeneration—To compare the time course of dephosphorylation and that of pigment regeneration, we measured time course of pigment regeneration in rod and cone membranes spectrophotometrically under the conditions similar to that used for the measurement of dephosphorylation activities. In this pigment regeneration measurement, NH$_4$OH was not added, so that late bleaching intermediates such as metarhodopsin III could affect the spectrophotometric quantification of the regenerated pigment. It is not known whether such intermediates are present for a relatively long time after a light flash in the presence of 11-cis retinal. To minimize the contribution of metarhodopsin III ($\lambda_{max}$~450 nm) and that of N-retinylidene-phosphatidylethanolamine ($\lambda_{max}$~450 nm), if any, in the case of rod pigment, regenerated rod pigment ($\lambda_{max}$~500 nm) was quantified at 550 nm using a rapid scanning spectrophotometer, USB4000 equipped with DT-MINI-2-GS light source (Ocean Optics). The data of the spectrum in the visible region was collected at the desired time. In the case of cone pigments, regeneration of cone pigment is very fast, so that the spectrum was recorded every 250 ms. To minimize the effect of metarhodopsin III and that of N-retinylidene-phosphatidylethanolamine and also for a technical reason that only the red-sensitive pigment can be quantitated in a mixture of various types of cone pigments, regenerated red-sensitive cone pigment was quantified at 570 nm in cone membranes. The bleach of the pigment by measuring light was <0.3% during the measurements of rod and cone pigment regeneration.

Results and Discussion

Dephosphorylation in BI-P in Rod and Cone Membranes—To compare the rate of dephosphorylation between rods and cones, we first measured time-dependent changes in pigment phosphorylation levels in the presence of ATP after a light flash in rod and cone membranes. In rod membranes, the phosphorylation level of light-activated visual pigment continuously increased (Fig. 1A, open circles), and apparent dephosphoryla-

**FIGURE 1. Time courses of phosphorylation levels in BI-P after a light flash in rod and cone membranes.** Shown are time-dependent changes in phosphorylation levels in BI-P after a light flash in rod and cone membranes in the absence and presence of the cytoplasmic proteins. The level of phosphorylation is expressed as the number of phosphates ($P_1$) incorporated into an activated visual pigment ($R^*$. A, time courses in rod membranes. B, time courses in cone membranes. The inset in B shows initial phase of the time course in cone membranes. In A and B, open symbols indicate the results without the addition of the cytoplasmic proteins of rods and cones, respectively, and filled symbols indicate those with the addition of (+ cyto). Final protein concentration of the rod cytoplasmic proteins added was 4 mg/ml in rod membranes (A), and that of the cone cytoplasmic proteins was 39 mg/ml in cone membranes (B). Each data point is shown as the mean ± S.D. (n = 3).
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The rate of dephosphorylation in bleached intermediate (BI-P) in the study shown in Fig. 1 was difficult to determine correctly. This was because phosphorylation in addition to dephosphorylation was taking place, and relative contribution of each of the reactions was difficult to determine. However, under the assumption that the phosphorylation can be neglected at times after the peak of phosphorylation (>10 min in rod membranes and >5 s in cone membranes), the initial rates of dephosphorylation in the presence of the cytoplasmic proteins (4 mg/ml in rod membranes and 39 mg/ml in cone membranes) were 0.019 ± 0.009 Pi dissociated per R* per min (P/R*/min) (n = 3) in rod membranes and 0.42 ± 0.16 P/R*/min (n = 3) in cone membranes. These values are the lower limits of the dephosphorylation activities in rod and cone membranes under the conditions employed.

The increase in dephosphorylation activities in the presence of the cytoplasmic proteins is probably due to the presence of phosphatase(s) in these proteins, not the factors that increase the phosphatase activity. It is because even after treatment of rod or cone membranes with 5 M urea to denature the intrinsic phosphatase activities completely, the addition of the cytoplasmic proteins increased the dephosphorylation activity.

Estimation of Dephosphorylation Activities in Opsin-P in Living Rods and Cones—As shown above, cytoplasmic proteins prepared from our purified rods and cones contained the activity of dephosphorylation of BI-P, which is probably attained by phosphatases in the outer segment. We tried to estimate this activity in living cells. For this purpose, we determined the concentration of the cytoplasmic proteins containing outer segment proteins at a concentration similar to that in living cells (CCP/OSP). For this purpose we first quantified the amount of cytoplasmic proteins present in known numbers of rod or cone cells and obtained the content of cytoplasmic proteins in a rod and a cone as an average: they were 7.2 ± 0.5 pg/rod (n = 3) and 188 ± 59 pg/cone (n = 3). Our purified rods and cones consist of the outer segment and the inner segment and are devoid of nucleus and terminal regions (for example, see Fig. 2 in Ref. 1).

To obtain estimates of the CCP/OSP, we assumed that the cytoplasmic volume in the outer segment of a rod and a cone is half of the outer segment envelope volume (27), which is the volume determined from the outer dimension of an outer segment. In case responsible proteins for dephosphorylation are localized only in the outer segment, the CCP/OSP can be calculated by dividing the above protein content in a single rod or cone (7.2 pg/rod and 188 pg/cone) by half of the outer segment envelope volume. In this case, based on our volume measurements from the outer dimension of the outer segment of a rod and a cone (Table 1), we obtained the value of 390 ± 30 mg/ml for rod cytoplasmic proteins and 6.5 ± 2.0 g/ml for cone cytoplasmic proteins as the CCP/OSP.

In the case where responsible proteins for dephosphorylation distribute both in the outer segment and the inner segment evenly, we need to consider the cytoplasmic volume of the inner segment. When we assume that the cytoplasmic volume of the inner segment is a half of the envelope volume as in the outer segment, based on our estimation of the inner segment volume (Table 1), we obtained the CCP/OSP of 297 ± 105 mg/ml in a rod and 959 ± 316 mg/ml in a cone. We also estimated the CCP/OSP in the case where the cytoplasmic volume in the inner segment is 70% of the inner segment envelope volume. In this case, the concentrations were 250 ± 20 mg/ml in rods and 570 ± 180 mg/ml in cones. Although it is difficult to estimate the CCP/OSP correctly, we believe that our above estimation

### Table 1

| Cell type | Cytoplasmic proteins | Volume |
|-----------|----------------------|--------|
|           | pg/cell              | Outer segment | Inner segment |
| Rod       | 7.2 ± 0.5 (n = 3)    | 37 ± 4 (n = 22) | 11 ± 2 (n = 22) |
| Cone      | 188 ± 59 (n = 3)     | 58 ± 6 (n = 21) | 335 ± 24 (n = 21) |

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**Note:**
- The table provides the estimated concentrations of cytoplasmic proteins in rod and cone membranes.
- The values indicate the amount of cytoplasmic proteins per unit volume of the outer segment and inner segment.
- The concentrations are given in mg/ml.
- The values are based on the assumption of the cytoplasmic volume in the outer segment and inner segment.
- The data are given for both rods and cones, with respective concentrations and standard deviations for each case.
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FIGURE 2. Does-dependent effect of the cytoplasmic proteins on dephosphorylation in Opsin-P. Shown is dephosphorylation in Opsin-P in rod membranes (A) and in cone membranes (B) in the presence of their cytoplasmic proteins at various concentrations indicated (mg/ml). The level of dephosphorylation is expressed as the number of phosphates in an opsin molecule (P/opsin). Each data point in A and B is shown as the mean ± S.D. (n = 3); C, dose-dependent effect of the cytoplasmic proteins on the initial dephosphorylation rate constant (see “Experimental Procedures”) in rod and cone membranes. Initial rates of dephosphorylation (P/opsin) obtained in A and B are plotted against the protein concentration of the cytoplasmic proteins in rod (open circles) and cone (filled triangles) membranes. Each data point is shown as the mean ± S.D. (n = 3), except that obtained at 150 mg/ml protein concentration in cone membranes; it was examined only one time because of limitation of availability of cone material. The results were fitted to the Michaelis-Menten equation for rod (dotted curve) and cone (solid curve) membranes. The fitted $K_m$ and $V_{max}$ values in rod membranes were 2.7 mg/ml and 0.16 P, released from an opsin molecule per min (P/opsin/min), respectively, and those in cone membranes were 17 mg/ml and 0.47 P/opsin/min. Data at 0.4 and 10 mg/ml in rod membranes and those at 0.4 mg/ml in cone membranes are not shown in A and B, respectively, to avoid crowding.

In Fig. 1 we measured dephosphorylation after giving a light flash in BI-P and estimated the lower limit of the phosphorylation rate in the presence of the cytoplasmic proteins. In Fig. 2, we measured dephosphorylation in Opsin-P in the presence of the cytoplasmic proteins. At a similar concentration of the added cytoplasmic proteins, we can compare the rate of dephosphorylation at two different stages in the visual pigment regeneration cycle. In rod membranes, the initial dephosphorylation rates were 0.019 ± 0.009 P/R$^*/$min minimally in BI-P (4 mg/ml cytoplasmic proteins in Fig. 1A) and 0.052 ± 0.016 P/opsin/min (n = 3) in Opsin-P (2 mg/ml cytoplasmic proteins in Fig. 2A). In cone membranes, they were 0.42 ± 0.16 P/R$^*/$min in BI-P (39 mg/ml in Fig. 1B) and 0.37 ± 0.04 P/opsin/min (n = 3) in Opsin-P (50 mg/ml in Fig. 2B). The rates are not so different between BI-P and Opsin-P in either rod or cone membranes. The result, therefore, suggests that the dephosphorylation rate is not so different between BI-P and Opsin-P.

In a separate study we measured the initial rate of dephosphorylation activity in rod membranes with rod cytoplasmic proteins and also the rate in rod membranes with the cone cytoplasmic proteins, both at 2 mg/ml. The initial rate was higher in the presence of the cone proteins than the rod proteins (0.056 ± 0.006 P/opsin/min with cone proteins and 0.035 ± 0.003 P/opsin/min with rod proteins, n = 3). Taking the difference in the $K_m$ values into consideration (2.7 mg/ml for the rod proteins and 17 mg/ml for the cone proteins), the result suggests that phosphatase(s), not Opsin-P, is responsible for the higher dephosphorylation activity in cones.

Measurements of Dephosphorylation in VP-P—Dephosphorylation was measured in VP-P in the dark and compared to that in Opsin-P in both rod (Fig. 3, A and B) and cone membranes determined (see “Experimental Procedures”). In Fig. 2C, each initial rate in rod (open circles) or cone membranes (filled triangles) was plotted against the concentration of the cytoplasmic proteins, and the results were fitted with the Michaelis-Menten equation. The estimated $K_m$ and $V_{max}$ in rod membranes were 2.7 mg/ml of the rod cytoplasmic proteins and 0.16 P, dissociated from opsin per min (P/opsin/min), respectively. In cone membranes they were 17 mg/ml and 0.47 P/opsin/min. Our estimates of the CCP/OSP, the concentration of the cytoplasmic proteins to exert their in vivo effects, were 250–390 mg/ml in rod membranes and 570 mg/ml to 6.5 g/ml in cone membranes (see above) and were >100 and >35 times higher than the $K_m$ values in rod and cone membranes, respectively. For this reason, it is probable that dephosphorylation reaction exerts its maximum activity ($V_{max}$; 0.16 P/opsin/min in rod membranes and 0.47 P/opsin/min in cone membranes) in living rods and cones. Half-times of dephosphorylation expected from the $V_{max}$ values of the initial rate of dephosphorylation were 4.3 min in rod membranes and 1.5 min in cone membranes. However, the actual half-times of dephosphorylation we obtained in Fig. 2 were much longer than these, probably because Opsin-P was multiply phosphorylated. The actual half-times in rod membranes (~64 min at 50 mg/ml cytoplasmic proteins in Fig. 2A) and in cone membranes (~13.5 min at 150 mg/ml cytoplasmic proteins in Fig. 2B) are broadly similar to those observed in living animals, ~50 min in mouse rods (12, 13) and 4 min in zebrafish (14).

gives us the range of the CCP/OSP that enables us to consider the in vivo effect of the cytoplasmic proteins on dephosphorylation: 250–390 mg/ml in rod membranes and 570 mg/ml to 6.5 g/ml in cone membranes.

To evaluate the phosphatase activity in living rods and cones, dephosphorylation time course was measured in membranes containing Opsin-P in the presence of the cytoplasmic proteins of various concentrations in rod (Fig. 2A) and cone (Fig. 2B) membranes. In these sets of measurements, dephosphorylation activities in the absence of the cytoplasmic proteins in rod membranes (0 mg/ml in Fig. 2A) and in cone membranes (0 mg/ml in Fig. 2B) were similar, which is probably because phosphatase activity in cone membranes was eluted significantly in this study. From curve fitting to the double exponential function, the values of the initial rate of dephosphorylation were

- $K_m$: Michaelis-Menten constant
- $V_{max}$: maximum velocity of the enzyme reaction
- $P$: phosphatase activity
- $R^*$: retinal
- $opsin$: opsins
- $mg/ml$: milligrams per milliliter
Dephosphorylation of Visual Pigment in Rods and Cones

Up to here, we examined dephosphorylation in BI-P (Fig. 1), Opsin-P (Figs. 2 and 3), and VP-P (Figs. 3 and 4). In living cells, bleached pigment is regenerated with binding to 11-cis retinal supplied through a conventional retinoid cycle to rods and cones and through a cone-specific retinoid cycle to cones (3). It could be the case that pigment regeneration couples with the dephosphorylation reaction. To test this possibility, we examined the effect of pigment regeneration on a dephosphorylation reaction in BI-P. First, we examined the effect of bleaching on dephosphorylation in VP-P (open symbols in Fig. 4, A and B) and in the presence (2 mg/ml proteins, B and E) of the cytoplasmic (cyto) proteins. Their dephosphorylation time courses were compared with those obtained in VP-P kept in the dark (filled symbols and dotted curves). Each data point shown in A, B, D, and E is the mean ± S.D. (n = 3). Initial rates of dephosphorylation (P/R in VP-P kept in the dark and P/R* in VP-P activated by a light flash) were determined and are shown for rod membranes in C and for cone membranes in F (both, n = 3).

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Effect of Pigment Regeneration on Dephosphorylation in Rod and Cone Membranes—Up to here, we examined dephosphorylation in BI-P (Fig. 1), Opsin-P (Figs. 2 and 3), and VP-P (Figs. 3 and 4). In living cells, bleached pigment is regenerated with binding to 11-cis retinal supplied through a conventional retinoid cycle to rods and cones and through a cone-specific retinoid cycle to cones (3). It could be the case that pigment regeneration couples with the dephosphorylation reaction. To test this possibility, we examined the effect of pigment regeneration on a dephosphorylation reaction in BI-P. First, we examined the effect of bleaching on dephosphorylation in VP-P (open symbols in Fig. 4, A and B) and in the presence (2 mg/ml proteins, B and E) of the cytoplasmic (cyto) proteins. Their dephosphorylation time courses were compared with those obtained in VP-P kept in the dark (filled symbols and dotted curves). Each data point shown in A, B, D, and E is the mean ± S.D. (n = 3). Initial rates of dephosphorylation (P/R in VP-P kept in the dark and P/R* in VP-P activated by a light flash) were determined and are shown for rod membranes in C and for cone membranes in F (both, n = 3).
Dephosphorylation of Visual Pigment in Rods and Cones

Dephosphorylation was measured in the presence of pigment regeneration (\(+11\text{cRAL}\)) in rod membranes (Fig. 5, B and C) and in cone membranes (Fig. 5, E and F) in the absence (Fig. 5, B and E) and presence of the cytoplasmic proteins (5 mg proteins/ml in rod membranes in Fig. 5C and 20 mg proteins/ml in cone membranes in Fig. 5F). As controls, we added all-trans retinal (\(+atRAL\)) instead of 11-cis retinal. Although pigment regeneration seems to inhibit phosphorylation in rods (most effectively shown in Fig. 5B), the addition of 11-cis retinal (filled symbols) and, therefore, pigment regeneration did not affect the dephosphorylation significantly in the absence (Fig. 5B) and presence (Fig. 5C) of the cytoplasmic proteins in rod membranes. Similarly, pigment regeneration did not affect the dephosphorylation in cone membranes (Fig. 5, E and F).

Effects of the Cytoplasmic Proteins on Dephosphorylation—In this study we found that the phosphatase activity on phosphorylated visual pigment is low in rod and cone membranes alone, and the addition of the cytoplasmic proteins extracted from purified rods and cones increased the activity (Fig. 1). The increase in the activity should be due to the presence of the phosphatase activity, not the presence of activating factors (see above). We added all of the cytoplasmic proteins in the outer segment and the inner segment. So far, it is not known whether phosphatases probably present in the rod and cone inner segment can dephosphorylate BI-P, Opsin-P, or VP-P, and this study did not add any information on this point. If phosphatases in the inner segment show activities on phosphorylated pigment, phosphatase activities in the outer segment of a rod and a cone are lower than those estimated in Fig. 2.

It has been reported that protein phosphatase 2A is present in bovine rod outer segments (15). Our control study showed that 20 \(\mu\)M okadaic acid, an inhibitor of the concentration that suppresses protein phosphatase 1 (PP1), PP2A, PP2B, and PP5 (28), inhibited exogenously applied PP2A in BI-P in mouse rod membranes, but it was not effective on our carp rod or cone membranes in the presence of the cytoplasmic proteins. Furthermore, chelation of Mg\(^{2+}\) by EDTA to inhibit PP2C was not effective. We also tried to separate the phosphatase activity, but we lost the activity during chromatographic separation. As a consequence, our attempt to identify the phosphatase(s) responsible for dephosphorylation in our study was not successful.

Arrestin has been reported to inhibit dephosphorylation (24). We measured dephosphorylation in the presence of the rod and cone cytoplasmic proteins, which contained rod arrestins (rArr1 and rArr2) and cone arrestins (cArr1 and cArr2), respectively. Carp rod and cone arrestins bind to bleached pigment no matter whether the pigment is phosphorylated or not (5), which suggests that arrestins bind essentially to bleached pigment. In agreement with this notion, it has been shown that bovine rod arrestin does not bind to VP-P in the dark (29). In our present study dephosphorylation activity was similar between VP-P kept in the dark and that bleached by light (Fig. 4) in the presence of the cytoplasmic proteins or arrestins in both rod and cone membranes. The result, therefore, suggests that arrestins do not affect dephosphorylation in rods or cones as implied previously in mouse rods (13) or that the inhibition of dephosphorylation by arrestin and the facilitation of dephos-

![Figure 5. Effect of pigment regeneration on dephosphorylation in BI-P.](image)

- **A**: time course of pigment regeneration in carp rod membranes. Rod membranes were bleached with a light flash in the presence of ATP and 11-cis retinal. Subsequent pigment regeneration in the dark was measured at 550 nm at the time indicated after the bleach (see “Experimental Procedures”). The fitted curve shows an exponential function with a half-time of 121 s with a delay time of 1.2 s.
- **B**: time-dependence changes in pigment phosphorylation levels measured in rod membranes in the dark supplemented with all-trans retinal (open circles) or 11-cis retinal (filled circles). The level of pigment phosphorylation is expressed as the number of incorporated phosphates per R* (P/R*).
- **C**: time-dependent changes in pigment phosphorylation levels measured in cone membranes similarly as in B but in the presence of the rod cytoplasmic proteins (5 mg/ml). D: time course of pigment regeneration in carp cone membranes. Cone membranes were bleached with a light flash in the presence of ATP and 11-cis retinal. Subsequent pigment regeneration in the dark was measured by monitoring the increase in the absorption at 570 nm, the absorption maximum of regenerated red-sensitive cone pigment. The fitted curve shows an exponential function with a half-time of 8.1 s with a delay time of 8.1 s.
- **E**: time-dependent changes in pigment phosphorylation levels measured in rod membranes supplemented with all-trans retinal (open triangles) or 11-cis retinal (filled triangles). The level of pigment phosphorylation is expressed as the number of phosphates incorporated per R* (P/R*).
- **F**: time-dependent changes in pigment phosphorylation levels measured in cone membranes similarly as in E but in the presence of the cone cytoplasmic proteins (20 mg/ml). Each data point shown is the mean ± S.D. \((n = 3)\).
Dephosphorylation of Visual Pigment in Rods and Cones

Dephosphorylation by phosphatase(s) are almost equally balanced with the addition of the cytoplasmic proteins. A further study is required to determine which is the case.

Physiological Relevance of the Biochemical Measurements of Dephosphorylation and Pigment Regeneration—In the present study, half-times of dephosphorylation reaction in Opsin-P, for example, were ~64 min in rod membranes and ~13.5 min in cone membranes in the presence of the cytoplasmic proteins at the maximum concentrations we examined (50 mg/ml in rod membranes and 150 mg/ml in cone membranes; Fig. 2). These half-times are close to those obtained previously in living animals under the condition of high bleach levels as in this study: ~50 min in mouse rods (12, 13) and 4 min in zebrafish cones (14). From these results we could say that our biochemical measurements of dephosphorylation reaction are physiologically relevant; the range of the time required for half-reduction of the phosphorylation levels is 50–60 min in rods and 4–15 min in cones in living animals at high bleach levels.

Our present study showed that pigment regeneration in membrane preparations is fast; half-times required for pigment regeneration were ~120 s in rod membranes and ~8 s in cone membranes (Fig. 5, A and D) even though 65 and 80% pigment were bleached in rod and cone membranes, respectively. In contrast, under similar high bleach conditions in rods and cones in living animals, half-times are much longer, and they have been reported to be 50–60 min in mouse (12, 13) and frog rods (30) and 22 s in frog red-sensitive cones (31). In our measurement in membranes, we added an excess amount of 11-cis retinal, which is probably the reason why the pigment regeneration in our biochemical study is much faster than that in living animals. In other words the supply of 11-cis retinal is probably limited so that the regeneration is slower in living animals.

We showed that pigment regeneration does not affect dephosphorylation in either rod or cone membranes (Fig. 5). However, it has been shown that dephosphorylation and pigment regeneration show similar time courses in rods in living mouse (12). This result could be explained by assuming that the supply of 11-cis retinal is the limiting step in living rods. Because of this limitation, pigment regeneration would be slow in living rods, so that the time course of pigment regeneration is similar to that of dephosphorylation. It is also reported that Rlbp−/− mice, in which pigment regeneration is expected to be slower than that in wild-type mice, showed normal dephosphorylation time course (12). This result is consistent with the above notion that the supply of 11-cis retinal is the limiting step of pigment regeneration in living rods.

Visual Pigment Dephosphorylation during Its Regeneration Cycle—In this study we showed that the rate of dephosphorylation is not different much among the stages in the visual pigment regeneration cycle in the presence of the cytoplasmic proteins; dephosphorylation activities were similar in BI-P, Opsin-P, and VP-P in both rod and cone membranes (Figs. 1, 3, and 4). In addition, dephosphorylation activities were not affected by pigment regeneration (Fig. 5). All of these findings suggest strongly that pigment dephosphorylation takes place constantly in the pigment regeneration cycle. In vivo pigment dephosphorylation activities would be higher in cones than in rods (Fig. 2), which will probably be advantageous for cones to maintain the level of functional visual pigment even under bright light (but see below).

In the case of cones in living cold-blooded animals, pigment regeneration (half-time, 22 s in Ref. 31) seems to be faster than dephosphorylation (half-time, 4–15 min, see above) at least by a factor of 10. This comparison strongly suggests that in cones, some of the regenerated pigments remain phosphorylated. This possibility has been suggested previously in living mouse rods (32), but the extent of the presence of VP-P could be much larger in cones, because time courses are similar in pigment regeneration and in dephosphorylation in rods (12), whereas pigment regeneration is much faster than dephosphorylation in cones as shown above. Our measurement of these time courses in rod and cone membranes (Fig. 5) supports this view. Population of VP-P would depend on both how much pigment is bleached in unit time and the rate of supply of 11-cis retinal, but surely it is high under bright light. It has been shown that bleached VP-P activates the phototransduction cascade much less effectively than the non-phosphorylated pigment (33). In agreement with this previous study, we observed a 3.7-times decrease in the rate of transducin activation in VP-P compared with that in regenerated and non-phosphorylated pigment (VP) in rod membranes; the rates were 20.5 ± 8.1 transducin activated per R* per s (Tr*2/R*/s) (n = 3) in VP and 5.6 ± 3.6 Tr*/R*/s in VP-P (n = 3). In cone membranes, the rate was 11× lower in VP-P; they were 4.1 ± 1.3 Tr*/R*/s in VP (n = 3) and 0.37 ± 0.23 Tr*/R*/s in VP-P (n = 3). For this reason, VP-P should have some roles in desensitization in cones during light adaptation.

Author Contributions—H. Y. designed, performed, and analyzed the experiments and wrote the paper. S. T. and S. K. designed the study and analyzed the experiments and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

References

1. Kawamura, S., and Tachibana, S. (2014) Phototransduction in rods and cones. in Vertebrate Photoreceptors: Functional Molecular Bases (Furukawa, T., Hurley, J. B., and Kawamura, S., eds) pp. 23–45, Springer, Tokyo, Japan
2. Yau, K. W., and Hardie, R. C. (2009) Phototransduction motifs and variations. Cell 139, 246–264
3. Tanguy, P. H., Kono, M., Koutalos, Y., Ablonycz, Z., and Crouch, R. K. (2013) New insights into retinoid metabolism and cycling within the retina. Prog. Retin. Eye Res. 32, 48–63
4. Arshavsky, V. Y. (2002) Rhodopsin phosphorylation: from terminating single photon responses to photoreceptor dark adaptation. Trends Neurosci. 25, 124–126
5. Tomizuka, J., Tachibana, S., and Kawamura, S. (2015) Phosphorylation-independent suppression of light-activated visual pigment by arrestin in carp rods and cones. J. Biol. Chem. 290, 9399–9411
6. Burkhardt, D. A. (1994) Light adaptation and photopigment bleaching in cone photoreceptors in situ in the retina of the turtle. J. Neurosci. 14, 1091–1105
7. Jones, G. J., Crouch, R. K., Wiggert, B., Cornwall, M. C., and Chader, G. J. (1989) Retinoid requirements for recovery of sensitivity after visual-pigment bleaching in isolated photoreceptors. Proc. Natl. Acad. Sci. U.S.A. 86, 9606–9610
8. Mata, N. L., Radu, R. A., Cleemans, R. C., and Travis, G. H. (2002) Isomerization and oxidation of vitamin A in cone-dominant retinas: a novel path-
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way for visual-pigment regeneration in daylight. Neuron 36, 69–80
9. Ala-Laurila, P., Cornwall, M. C., Crouch, R. K., and Kono, M. (2009) The action of 11-cis-retinol on cone opsins and intact cone photoreceptors. J. Biol. Chem. 284, 16492–16500
10. Miyazono, S., Shimauchi-Matsukawa, Y., Tachibanaki, S., and Kawamura, S. (2008) Highly efficient retinal metabolism in cones. Proc. Natl. Acad. Sci. U.S.A. 105, 16051–16056
11. Kühn, H. (1974) Light-dependent phosphorylation of rhodopsin in living frogs. Nature 250, 588–590
12. Ohguro, H., Van Hooser, J. P., Milam, A. H., and Palczewski, K. (1995) Rhodopsin phosphorylation and dephosphorylation in vivo. J. Biol. Chem. 270, 14259–14262
13. Kennedy, M. J., Lee, K. A., Niemi, G. A., Craven, K. B., Garwin, G. G., Saari, J. C., and Hurley, J. B. (2001) Multiple phosphorylation of rhodopsin and the in vivo chemistry underlying rod photoreceptor dark adaptation. Neuron 31, 87–101
14. Kennedy, M. J., Dunn, F. A., and Hurley, J. B. (2004) Visual pigment phosphorylation but not transducin translocation can contribute to light adaptation in zebrafish cones. Neuron 41, 915–928
15. Palczewski, K., Hargrave, P. A., McDowell, J. H., and Ingebritsen, T. S. (1989) The catalytic subunit of phosphatase 2A dephosphorylates phosphoprotein. Biochemistry 28, 415–419
16. Ohguro, H., Palczewski, K., Ericsson, L. H., Walsh, K. A., and Johnson, R. S. (1993) Sequential phosphorylation of rhodopsin at multiple sites. Biochemistry 32, 5718–5724
17. Tachibanaki, S., Arinobu, D., Shimauchi-Matsukawa, Y., Tsushima, S., and Kawamura, S. (2005) Highly effective phosphorylation by G protein-coupled receptor kinase 7 of light-activated visual pigment in cones. Proc. Natl. Acad. Sci. U.S.A. 102, 9329–9334
18. Tachibanaki, S., Tsushima, S., and Kawamura, S. (2001) Low amplification and fast visual pigment phosphorylation as mechanisms characterizing cone photoreceptor responses. Proc. Natl. Acad. Sci. U.S.A. 98, 14004–14009
19. Brown, P. K., Gibbons, I. R., and Wald, G. (1963) The visual cells and visual pigment of the mudpuppy, Necturus. J. Cell Biol. 19, 79–106
20. Okano, T., Fukada, Y., Artamonov, I. D., and Yoshizawa, T. (1989) Purification of cone visual pigments from chicken retina. Biochemistry 28, 8848–8856
21. Kawamura, S. (1993) Rhodopsin phosphorylation as a mechanism of cyclic GMP phosphodiesterase regulation by S-modulin. Nature 362, 855–857
22. Kelleher, D. J., and Johnson, G. L. (1988) Transducin inhibition of light-dependent rhodopsin phosphorylation: evidence for βγ subunit interaction with rhodopsin. Mol. Pharmacol. 34, 452–460
23. Tachibanaki, S., Yonetsu, S., Fukaya, S., Koshitani, Y., and Kawamura, S. (2012) Low activation and fast inactivation of transducin in carp cones. J. Biol. Chem. 287, 41186–41194
24. Palczewski, K., McDowell J. H., Lakes, S., Ingebritsen, T. S., and Hargrave, P. A. (1989) Regulation of rhodopsin dephosphorylation by arrestin. J. Biol. Chem. 264, 15770–15773
25. Kutuzov, M. A., and Bennett, N. (1996) Calcium-activated opsin phosphatase activity in retinal rod outer segments. Eur. J. Biochem. 238, 613–622
26. Brown, B. M., Carlson, B. L., Zhu, X., Lolley, R. N., and Craft, C. M. (2002) Light-driven translocation of the protein phosphatase 2A complex regulates light/dark dephosphorylation of phosducin and rhodopsin. Biochemistry 41, 13526–13538
27. Peet, J. A., Bragin, A., Calvert, P. D., Nikonov, S. S., Mani, S., Zhao, X., Besharse, J. C., Pierce, E. A., Knox, B. E., and Pugh, E. N., Jr. (2004) Quantification of the cytoplasmic spaces of living cells with EGFP reveals arrestin-EGFP to be in disequilibrium in dark adapted rod photoreceptors. J. Cell Sci. 117, 3049–3059
28. Armstrong, C. G., Mann, D. J., Berndt, N., Cohen, P. T. (1995) Drosophila PPy, a novel male specific protein serine/threonine phosphatase localized in somatic cells of the testis. J. Cell Sci. 108, 3367–3375
29. Gurevich, V. V., Hanson, S. M., Song, X., Vishnevskiy, S. A., and Gurevich, E. V. (2011) The functional cycle of visual arrestins in photoreceptor cells. Proc. Retin. Eye Res. 30, 405–430
30. Peskin, J. C. (1942) The regeneration of visual purple in the living animal. J. Gen. Physiol. 26, 27–47
31. Taylor, J. W. (1969) Cone and possible rod components of the fast photoresponse in the frog eye: a new method of measuring cone regeneration rates in vivo. Vision Res. 9, 443–452
32. Lee, K. A., Nawrot, M., Garwin, G. G., Saari, J. C., and Hurley, J. B. (2010) Relationships among visual cycle retinoids, rhodopsin phosphorylation, and phototransduction in mouse eyes during light and dark adaptation. Biochemistry 49, 2454–2463
33. Wilden, U. (1995) Duration and amplitude of the light-induced cGMP hydrolysis in vertebrate photoreceptors are regulated by multiple phosphorylation of rhodopsin and by arrestin binding. Biochemistry 34, 1446–1454