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Different phosphorylation rates among vertebrate cone visual pigments with different spectral sensitivities

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ABSTRACT

Cone photoreceptor subtypes having different spectral sensitivities exhibit different recovery kinetics in their photoresponses in some vertebrates. Phosphorylation by G protein-coupled receptor kinase (GRK) is essential for the rapid inactivation of light-activated visual pigment, which is the rate-limiting step of the cone photoresponse recovery in salamander. In this study we compared the rate of light-dependent phosphorylation by GRK7 of carp green- and blue-sensitive cone visual pigments. Blue pigment was phosphorylated significantly less effectively than green pigment, suggesting that the difference in the pigment phosphorylation rate is responsible for the difference in photoresponse kinetics among cone photoreceptor subtypes.

1. Introduction

Vertebrate eyes have two types of photoreceptor cells, rods and cones. Rods mediate night vision and cones mediate daylight and color visions. Functional differences between rods and cones and their molecular mechanisms have been extensively investigated [1]. Although less studied, it is known that cone subtypes having different spectral sensitivities differ in their other properties, such as response sensitivity and kinetics. For example, it is reported that cone photoreceptors exhibit different kinetics of photoresponse recovery in amphibian [2] and fish [3,4] species. However, it is largely unknown what causes these differences.

Rod and cone photoreceptors contain visual pigments. Vertebrate visual pigments are classified into five groups, that is, four cone pigment groups [L (LWS), S (SWS1), M1 (SWS2), M2 (Rh2)] and one rhodopsin group [Rh (Rh1)], on the basis of amino acid sequence similarity [5–7]. This classification is well correlated with their absorption maximum wavelength (λ_{max}). It is well known that rhodopsin and cone pigments differ in some functional properties such as protein stability, photoreaction kinetics and regeneration rate [8]. On the other hand, differences in properties other than absorption wavelength among L, S, M1 and M2 groups are less investigated [8–10].

Phosphorylation of visual pigment by G protein-coupled receptor kinase (GRK) and subsequent binding of arrestin to the phosphorylated pigment are essential steps for the inactivation of photoactivated visual pigment and hence for the termination of phototransduction [11]. Recently, it was shown that the inactivation of the photoactivated visual pigment is the rate-limiting step of recovery of photoresponse in salamander cones at least for saturating responses [12,13]. This made us speculate that different rates of phosphorylation between cone pigments with different spectral sensitivities might be responsible for the difference in photoresponse recovery kinetics among cone subtypes.

In previous studies, light-dependent phosphorylation of cone visual pigments were biochemically observed with lizard [14], chicken [15], fish [16–18], and mammalian [19,20] cone pigments. However, to our knowledge, direct comparison of phosphorylation rates between cone pigments with different spectral sensitivities has never been reported.

In our previous studies we performed visual pigment phosphorylation assays with purified carp rods and cones [16,17]. However, it is difficult to purify separately cones with different spectral sensitivities. So in the present study, we prepared carp cone visual pigments by expression in HEK293T cells and compared their light-dependent phosphorylation rates by carp GRK7, which is specifically expressed in cones [17,21].

Abbreviations: λ_{max}, absorption maximum wavelength; GRK, G protein-coupled receptor kinase; UV, ultraviolet; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PC, L-α-phosphatidylcholine; Pi, inorganic phosphate.

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2. Materials and methods

2.1. Expression and purification of visual pigments

Red, green 1, green 2, blue and UV opsins (GenBank Accession Nos. are AB055656, AB110602, AB110603, AB113668 and AB113669, respectively) were cloned from the carp (*Cyprinus carpio*) retinal cDNA library [21]. The cloned cDNAs of carp cone opsins were tagged by the bovine rhodopsin 1D4 epitope sequence (ETSQVAPA) [22] at their C-terminal, unless noted otherwise. Then the cDNAs were introduced into an expression vector pCAGGS [23]. Opsins were expressed in the HEK293T cell by the calcium–phosphate method as previously reported [24]. After incubation of the transfected cells for 2 days, they were collected by centrifugation and suspended in buffer A (50 mM HEPES, 140 mM NaCl, 3 mM $MgCl_2$, pH 6.5). HEK293T cells expressing opsins were supplemented with 11-*cis*-retinal (final concentration: 20 μM) and incubated overnight to reconstitute the visual pigments. The following procedures were carried out under dim red light. The cell membranes were solubilized in buffer B (0.75% CHAPS, 10 mM HEPES, 115 mM K-gluconate, 2.5 mM KCl, 2 mM $MgCl_2$, 0.2 mM EGTA, 0.1 mM $CaCl_2$, 1 mM DTT, pH 7.5), and visual pigments were purified by adsorption on an anti-1D4-antibody-conjugated column and elution with buffer B containing 0.3 mg/mL 1D4 peptide.

2.2. Spectrophotometry

Absorption spectra of visual pigments were recorded at 4 °C by a Shimadzu UV2400 spectrophotometer.

2.3. Preparation of GRK7-expressing cell membrane

The cDNA of carp GRK7-1a (GenBank Accession No. is AB055658) [21] was introduced into an expression vector pCAGGS. GRK7 was expressed in the HEK293T cell by the calcium–phosphate method. Mevalonolactone was added to the culture medium (final concentration: 3 mM) right after the transfection in order to facilitate the C-terminal geranylgeranylation of GRK7. After incubation of the transfected cells for 2 days, they were collected by centrifugation and suspended in buffer A. GRK7-containing cell membranes were prepared by sucrose flotation as previously described [25] with some modifications. Briefly, the collected cells were homogenized in buffer A containing 8.6% (w/v) sucrose by using a homogenizer, and then layered on top of buffer A containing 40% (w/v) sucrose, followed by centrifugation in a swing-bucket rotor at 186,000×*g* for 1 h. The membranes floating at the interface between 8.6% and 40% sucrose layers were collected. The collected membranes were suspended in buffer C [10 mM HEPES, 115 mM K-gluconate, 2.5 mM KCl, 2 mM $MgCl_2$, 0.2 mM EGTA, 0.1 mM $CaCl_2$, 1 mM DTT, pH 7.5]. The amount of GRK7 in the membrane was quantified by SDS–PAGE and Coomassie blue staining with bovine serum albumin as the standard.

2.4. Reconstitution of visual pigments into liposome

Proteoliposomes containing visual pigments were prepared according to the previously reported method [26] with some modifications. Briefly, visual pigments were supplemented with a 500-fold molar excess of L-α-phosphatidylcholine (PC) dissolved in buffer B and incubated overnight. Then the samples were rapidly diluted by 5-fold with buffer C so that the CHAPS concentration was lowered to 0.15%, which was sufficiently lower than the critical micelle concentration (0.49%). The remaining monomeric CHAPS was removed by dialysis against a 20-fold excess volume of buffer C for ~24 h with four buffer exchanges. PC liposomes were collected by centrifugation at 100,000×*g* for 60 min and resuspended in buffer C. The aliquots of the liposome suspension were solubilized in buffer B and used for quantification of visual pigments in the liposomes by spectrophotometry. Visual pigment concentrations were calculated using the molar extinction coefficient of 40,000 M^{-1} cm^{-1} at the absorption maximum wavelength.

2.5. Phosphorylation assays

HEK293T cell membranes expressing GRK7 and PC liposomes containing visual pigment were mixed at an equivalent GRK:pigment molar ratio, frozen at −80 °C and then thawed to fuse the membranes right before the phosphorylation assays. Phosphorylation assays were performed at room temperature using a calibrated rapid-quench apparatus in which the timing of the addition of the reaction-stopping solution was controlled, as previously reported [17]. Fused membrane containing 12 pmol of visual pigment and 12 pmol of GRK7 (suspended in 15 μl of buffer C) were mixed with 10 μl of buffer C containing 2.5 mM ATP with tracer [γ-^{32}P]ATP, 1.25 mM GTP and additional 1.5 mM EGTA so that the mixture (25 μl) contained 1 mM ATP with tracer [γ-^{32}P]ATP, 0.5 mM GTP, 0.8 mM EGTA, 115 mM K-gluconate, 2.5 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM HEPES and 1 mM DTT. After preincubation for 30 s, the sample was irradiated with a white light flash which bleached ~50% of the pigment. The flash illumination was omitted for "dark" samples. The reaction was terminated at 0, 0.1, 0.2, 0.3, 0.5, 0.75 or 1 s after the flash by adding ~150 μl of 10% (w/v) trichloroacetic acid. After centrifugation (20,000×*g* for 1 h), the precipitate was washed with buffer C without DTT and subjected to SDS–PAGE. Then the amount of ^{32}P incorporated into the visual pigment band was quantified. The data of "dark" samples were subtracted from the data of "light" samples to calculate the light-dependent phosphorylation.

Phosphorylation assays of P189V mutants of green 1 and blue pigments were performed similarly, except that the reaction was terminated at 10 s after the flash.

3. Results

3.1. Expression of carp cone visual pigments

First, we cloned carp (*C. carpio*) red (L group), green 1 (M2 group), green 2 (M2 group), blue (M1 group) and UV (S group) cone visual pigments from retinal cDNA library [21] (GenBank Accession Nos. are AB055656, AB110602, AB110603, AB113668 and AB113669, respectively). Then we tagged them with the 1D4 epitope sequence (ETSQVAPA) at the C-terminal, cloned them into an expression vector and expressed in HEK293T cells. The pigment in transfected HEK293T cell membranes was regenerated with 11-*cis*-retinal, solubilized with CHAPS and affinity-purified by the 1D4 antibody. Bovine rhodopsin was also expressed, regenerated and purified as a control.

Absorption spectra of the purified expressed carp cone visual pigments and bovine rhodopsin are shown in Fig. 1. Green 1, green 2, blue and UV pigments exhibited their λ_{max} values at 510, 500, 445 and 370 nm, respectively. These values are in good agreement with the reported values for goldfish pigments (green 1: 511 nm, green 2: 506 nm, blue: 440 nm, UV: 359 nm) [27,28] which have 92–97% amino acid sequence identities to those of carp pigments. Unfortunately, the expression level of red pigment was not sufficient for obtaining a reliable absorption spectrum.

It should be noted that native carp visual pigments contain 11-*cis* 3,4-dehydroretinal (A2 11-*cis*-retinal), not A1 11-*cis*-retinal. Absorption spectra of carp cone visual pigments extracted from native carp photoreceptor membranes are known (λ_{max} values; red: 618 nm, green: 535 nm, blue: 460 nm) [16]. Absorption max-