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Multiple Visual Pigments in a Photoreceptor of the Salamander Retina

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ABSTRACT Although a given retina typically contains several visual pigments, each formed from a retinal chromophore bound to a specific opsin protein, single photoreceptor cells have been thought to express only one type of opsin. This design maximizes a cell's sensitivity to a particular wavelength band and facilitates wavelength discrimination in retinas that process color. We report electrophysiological evidence that the ultraviolet-sensitive cone of salamander violates this rule. This cell contains three different functional opsins. The three opsins could combine with the two different chromophores present in salamander retina to form six visual pigments. Whereas rods and other cones of salamander use both chromophores, they appear to express only one type of opsin per cell. In visual pigment absorption spectra, the bandwidth at half-maximal sensitivity increases as the pigment's wavelength maximum decreases. However, the bandwidth of the UV-absorbing pigment deviates from this trend; it is narrow like that of a red-absorbing pigment. In addition, the UV-absorbing pigment has a high apparent photosensitivity when compared with that of red- and blue-absorbing pigments and rhodopsin. These properties suggest that the mechanisms responsible for spectrally tuning visual pigments separate two absorption bands as the wavelength of maximal sensitivity shifts from UV to long wavelengths. Key words: spectral sensitivity • UV-sensitive cone • opsin • photosensitivity • vision

INTRODUCTION

The presence of more than one type of visual pigment in the retina broadens spectral sensitivity and provides the potential for wavelength discrimination. Visual pigments are composed of a protein, opsin, covalently linked to a retinal chromophore, which in vertebrates, is either 11-*cis* (A_1)[1] or 3-dehydro 11-*cis* retinal (A_2) (Goldsmith, 1990; Crescitelli, 1991). It is thought that individual photoreceptors express only one type of opsin (Goldsmith, 1990; Crescitelli, 1991) to maximize sensitivity to a given band of wavelengths and facilitate wavelength discrimination. For example, in short wavelength–sensitive cones of primates, the middle and long wavelength–sensitive pigments are present at fewer than one part in one hundred thousand (Baylor et al., 1987).

However, in some retinas, it may be desirable for individual photoreceptors to have a broad spectral sensitivity or for photoreceptors to adjust the wavelength range of their spectral sensitivity. Thus, a number of fish and amphibian photoreceptors contain a mixture of both A_1 and A_2 chromophores. In at least some cases the A_1/A_2 ratio may be subject to control; the chromophore may even be switched completely from one type to the other (Knowles and Dartnall, 1977*b*; Crescitelli, 1991; Bowmaker, 1995). The magnitude of the wavelength shift in spectral sensitivity induced by switching chromophores is somewhat limited, particularly for short wavelength–absorbing pigments (Knowles and Dartnall, 1977*a*; Harosi, 1994), but large changes could be attained by the expression of a different opsin. Evidence for the coexistence of two opsins in single photoreceptors has been obtained microspectrophotometrically (Shand et al., 1988; Archer and Lythgoe, 1990; Wood and Partridge, 1993) and immunohistochemically (Rohlich et al., 1994; Szel et al., 1994). We present evidence that the UV-sensitive cone of the salamander retina contains an unprecedented three opsins and show that all three form functional pigments (Makino et al., 1995).

METHODS

Tissue Preparation

Larval tiger salamanders (*Ambystoma tigrinum*, Charles Sullivan, Nashville, TN and Carl Lowrance, Tulsa, OK), 6–9 in in length, were cared for and used in accordance with institutional guidelines. In early experiments, salamanders were placed in darkness for 2–4 h just before use. Later, the minimum period of dark adaptation was extended to 12 h. After dark adaptation, the salamander was rapidly decapitated and pithed under dim red light. The retinas were removed and stored in Ringer's solution on ice. Periodically, a small sample was shredded and loaded into an experimental chamber under infrared illumination. The chamber was perfused continuously with Ringer's solution containing (mM): 115 NaCl, 2.5 KCl, 1.0 $MgCl_2$, 1.5 $CaCl_2$, 3.0 HEPES, 0.02 EDTA, 10 glucose, pH 7.6 at room temperature. In some experiments, the concentrations of HEPES and NaCl were 10 and 108 mM, respectively.

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[1]*Abbreviations used in this paper*: A_1, 11-*cis* retinal; A_2, 3-dehydro 11-*cis* retinal; ERC, early receptor current; i_o, flash strength giving rise to a half-maximal response; *P*, photosensitivity of pigment in solution; P_t, photosensitivity of pigment in the photoreceptor to transverse illumination with unpolarized light.

Published July 1, 1996

Spectral Sensitivity Determinations

The method of Baylor et al. (1984) was used with slight modifications. The inner segment of an isolated cell was drawn into a suction pipette. Membrane currents were recorded with a current-to-voltage converter (Axopatch 200; Axon Instruments, Foster City, CA), low-pass filtered at 30 Hz (−3 dB, eight-pole Bessel) and digitized at 200 Hz. Brief, unpolarized flashes were obtained by passing light from a shuttered incandescent or xenon arc source through an interference filter (nominal bandwidth 10 nm). An additional short wavelength–absorbing glass (GG435; Schott Glass Technologies, Inc., Duryea, PA) was inserted into the optical path during some trials. Flash strength was controlled with a series of neutral density filters. Generally, a flash of 10-ms nominal duration was used. However, flash duration was sometimes increased to 100 ms in an attempt to measure responses at wavelengths to which the cell was not very sensitive.

The method for determining spectral sensitivities is shown in Fig. 1. Responses to flashes at two or more strengths were recorded. The response amplitudes were then plotted against flash strength and fitted by eye with a Michaelis-Menten relation:

$$r/r_{max} = i/(i+i_o), \quad (1)$$

where r is the response amplitude, r_{max} is the maximal saturating response amplitude, i is the flash strength, and i_o is the flash strength giving rise to a half-maximal response. Measurements at a reference wavelength, usually 500 nm, were made frequently to monitor the state of the cell. Light passing through colored glass filters (GG475 or RG610; Schott Glass Technologies, Inc., Duryea, PA), containing wavelengths longer than 475 or 610 nm were used for partial bleaching experiments. Flash strength was calculated from measurements of the filtered light with a calibrated photometer (Graseby Optronics, Orlando, FL) after inserting its detector head into the optical path in place of the experimental chamber.

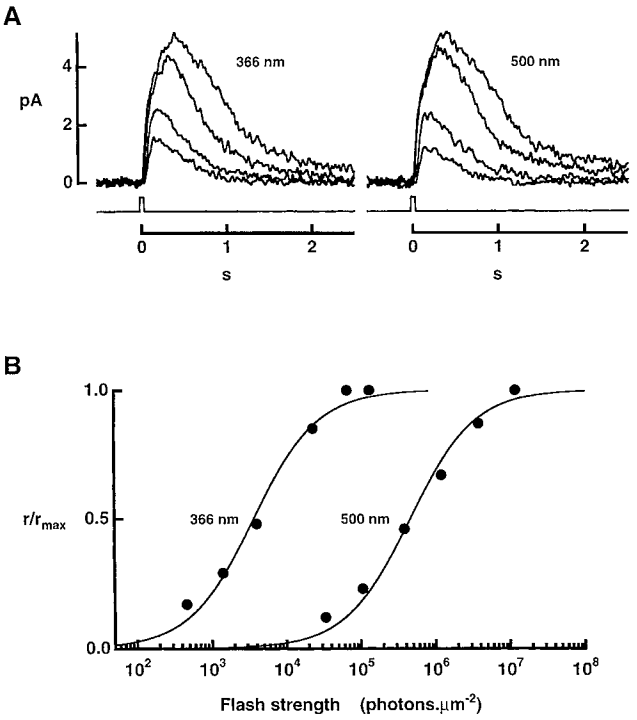

FIGURE 1. Univariance of a UV cone's flash responses. (*A*) Families of averaged responses from a UV cone to flashes at 366 and 500 nm. The amplitude of the saturating response was 5.2 pA. The lowermost trace of each panel shows the output of the flash monitor. (*B*) Stimulus–response relations. Symbols plot results from above, although traces corresponding to some points in the stimulus–response relations were omitted in *A* for clarity. Continuous lines show fits to Eq. 1 with an i_o of 3.30×10^3 photons·μm^{-2} at 366 nm and 4.10×10^5 photons·μm^{-2} at 500 nm.

Recordings of the maximal photocurrent ranged from 2 to 19 pA in red-sensitive cones (red cones), 9 to 45 pA in rods, 3 to 9 pA in blue-sensitive cones (blue cones), and 2 to 6 pA in UV-sensitive cones (UV cones). For salamanders that were dark adapted >16 h, i_o at the wavelength of maximal sensitivity was 1,630 ± 1,040 photons·μm^{-2} (mean ± SD, n = 16) for red cones, 5 ± 2 photons·μm^{-2} for rods (n = 16), 240 ± 120 photons·μm^{-2} for blue cones (n = 12), and 1,530 ± 1,210 photons·μm^{-2} for UV cones (n = 9).

The spectral sensitivities of three UV cones were determined from whole-cell voltage clamp recordings. Voltage clamp was established in two cells by rupturing the membrane beneath the patch and in the third by incorporation of the ionophore, nystatin. The membrane potential was clamped at −40 mV. The pipette contained (mM): 87 K^+ gluconate, 0.5 $MgCl_2$, 10 HEPES, 6 KCl, 5 EDTA, 0.02 EGTA, 1 K_2ATP, 1 Li_3GTP, and 0.18 nystatin (Sigma Chemical Co., St. Louis, MO), pH 7.2.

Sensitivity to wavelengths below 380 nm was not measured in all UV cones. However, eight complete UV cone spectra, normalized to their respective maxima, showed minimal variability at 400 nm. So, all UV cone spectra were normalized at 400 nm and averaged, and the resultant spectrum was plotted relative to its maximum.

A_1 chromophore was incorporated into cone pigment using the method of Jones et al. (1989). 50 μl l-α-phosphatidylcholine in chloroform (Sigma Chemical Co.) was dried under nitrogen and suspended in 500 μl Ringer's solution. The suspension was vortexed and then sonicated on ice until clear. An aliquot of a stock solution of A_1 in hexane was removed under dim red light and dried under nitrogen. Lipid vesicles were added to produce a final concentration of 0.5–10 mM retinal. The mixture was agitated slowly overnight at 5°C and then kept on ice until used. Photoreceptor outer segments were exposed to a bright step of light to release the native chromophore and were then perfused with A_1 in lipid vesicles.

The most bathochromically shifted native red and blue cone spectra and the spectra obtained after replacement of the cell's chromophore with A_1 were fitted with a sixth-order polynomial (Baylor et al., 1987):

$$\log S = \sum_{n=0}^{6} a_n \{\log[\lambda_{max}/(561\lambda)]\}^n, \quad (2)$$

where S is the sensitivity relative to that at the maximum, λ is wavelength, λ_{max} is the wavelength of maximal sensitivity, and the coefficients a_0 through a_6 are −5.2734, −87.403, 1228.4, −3346.3, −5070.3, 30881, and −31607. The most hypsochromically shifted native red and blue cone spectra were averaged and fitted with the log of the sum of the antilogs of the two polynomials for the bathochromic extreme native spectrum and the A_1 regenerated spectrum of each cone type where the relative contribution of each individual polynomial was the parameter of the fit. In calculating the proportions of each chromophore, it was assumed that the extinction coefficient for A_1 was 42,000 liters·mole^{-1}·cm^{-1} (Matthews et al., 1963) and that of A_2, 30,000 liters·mole^{-1}·cm^{-1} (Brown et al., 1963). Fits to native spectra were restricted to the long-wavelength side of the maximum since the short-wavelength side was not well described by the polynomial.

Published July 1, 1996

Photosensitivity Measurements

Isolated red and UV cones were stimulated with flashes from a xenon arc strobe (Strobex 236; Chadwick Helmuth, El Monte, CA) while under whole-cell voltage clamp, as described previously (Makino et al., 1991). Flashes were passed through a six-cavity interference filter of 440, 500, or 660 nm, whose bandwidth at half-maximal transmission was 30 nm (Omega Optical, Brattleboro, VT), or a near UV cut-on filter, KV380 or GG400 (50% transmission at 384 or 408 nm, respectively; Schott Glass Technologies, Inc.). The membrane potential was held between 0 and −50 mV, to minimize the holding current. The pipette contained (mM): 110 CsCl, 1 $MgCl_2$, 5 HEPES, and 1.0 EGTA. In some experiments the $MgCl_2$ concentration was raised to 2.5 or 5 mM. Currents were low-pass filtered at 5 or 10 kHz (−3 dB, eight-pole Bessel) and digitized at 20 kHz. Some digitized records were convolved with a Gaussian function for additional low-pass filtering at 1 kHz (−3 dB). Flashes elicited early receptor currents (ERC), rapid, capacitive current transients associated with the photoisomerization of visual pigment molecules, and a flash-induced artifact. The artifact was removed from the records as follows. Records of trials in which light was prevented from reaching the cell were averaged and subtracted from the average of all records of trials that elicited an ERC. This difference (ERC_{avg}) was then scaled to match the difference between each individual trial and the averaged artifact. The scaled ERC_{avg} was integrated starting from a point where the averaged artifact was zero. The sum of these integrals gives a lower limit for R_o, the total charge movement with complete bleach. P for red cone pigment was determined from the fit to:

$$q = R_o \exp(-P_t I)\,[\exp(P_t i_e) - 1], \qquad (3)$$

where q is the charge moved in response to a flash of effective strength i_e, P_t is the photosensitivity for side-on illumination with unpolarized light, I is the cumulative effective photon density to which the cell has been exposed before i_e (Makino et al., 1991). R_o and P_t were the parameters of the fit. P, for molecules oriented randomly in solution, was 1.33 P_t.

P for the UV cone was calculated from a component analysis. It was assumed initially that the photosensitivities and unitary charge movements were similar for the three pigments of this cell. Then from the averaged UV cone spectral sensitivity, 0.985 R_o was attributable to the UV-absorbing pigment, 0.015 R_o to the blue-absorbing pigment, and 0.000066 R_o to the red-absorbing pigment (Fig. 2). For each flash, Eq. 3 was first solved for the red- and blue-sensitive pigments individually using values for P_t of 5.4×10^{-9} $\mu m^2 \cdot molecule^{-1}$ and 5.8×10^{-9} $\mu m^2 \cdot molecule^{-1}$, respectively (Makino et al., 1991), to find q_R and q_B, the contributions of these two pigments to the observed q. These quantities, expressed as a product of R_o and a pigment-specific factor, f, were then subtracted from each observed q before using Eq. 3 to find P for the UV-sensitive pigment. To estimate the effective photons per flash for the UV pigment, it was assumed that the shape of this pigment's absorption spectrum followed Eq. 2, with a maximum located at 359 nm. The value thus obtained for the UV pigment P was used to reevaluate the relative proportions of the three pigments in the UV cone, and P was redetermined. After three iterations, the estimates of P ceased to change significantly.

RESULTS

Spectral Sensitivities of Salamander Photoreceptors

Salamander cones were classified by their relative sensitivity to flashes at different wavelengths into three categories: red-sensitive cones (red cones), blue-sensitive cones (blue cones), and UV-sensitive cones (UV cones) (Perry and McNaughton, 1991). Red cones, which were encountered most frequently (as observed by Perry and McNaughton, 1991), were morphologically heterogeneous; they appeared as large single, small single, or double cones (Mariani, 1986). Blue and UV cones always took the form of small single cones. Interestingly, UV cones sometimes had very small diameter outer segments (<1 μm).

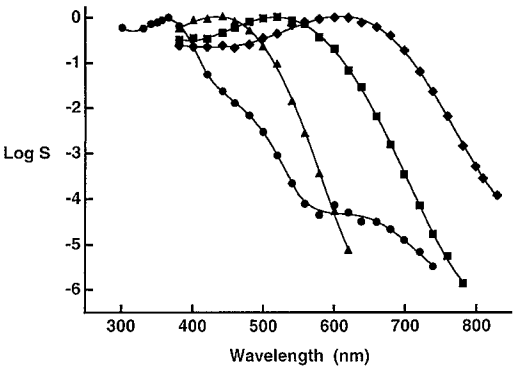

FIGURE 2. Spectral sensitivities of salamander photoreceptors. Symbols depict averages from 57 red cones (◆), 17 rods (■), 16 blue cones (▲), and 36 UV cones (●) (some results from Makino et al., 1990, 1991 have been included). Continuous curves were drawn by eye.

Flashes at different wavelengths elicited responses with similar kinetics in UV cones (Fig. 1 *A*). Negative responses, which could arise from electrical coupling to neighboring photoreceptors, were never observed for flashes between 300 and 740 nm. Thus, responses of UV cones obeyed univariance (Naka and Rushton, 1966), as did those of red cones, blue cones, and rods; wavelength influenced only the amount of light required to elicit the response, not its kinetics or polarity (Fig. 1). Collected spectral sensitivities of single salamander photoreceptors are shown in Fig. 2.

Although the spectral properties of tiger salamander photoreceptors have been studied in some detail (Harosi, 1975; Attwell et al., 1982; Cornwall et al., 1984; Makino et al., 1990; Perry and McNaughton, 1991), a feature not previously described is a sizable variation between individual spectra of a given type. The extreme cases are shown in Fig. 3 *A*. Spectral variation was greatest in red cones, where maxima spanned a 30–40-nm range. In rods (results not shown) and in blue cones, the range was 10–20 nm.

Approximately half of the red cones, rods, and blue cones exhibited the bathochromically extreme spectra. These spectra took the form expected for A_2 pigments (Ebrey and Honig, 1977; MacNichol, 1986). The hypsochromically extreme spectra were broader, suggestive of a chromophore mixture. Consistent with this scheme,

Published July 1, 1996

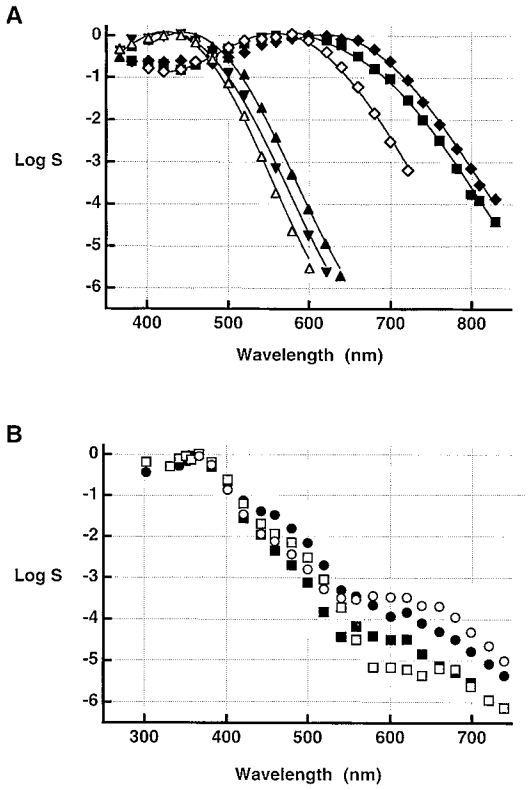

FIGURE 3. Individual variation in the spectral sensitivities. (*A*) Spectral sensitivities of red and blue cones with native (*filled symbols*) or A_1 chromophore (*open symbols*). Continuous lines show polynomial fits as described in Methods. Spectra with native chromophore fell between the bathochromic extreme (◆, red cone, $n = 28$; ▲, blue cone, $n = 5$) and the hypsochromic extreme spectra (■, red cone, $n = 4$; ▼, blue cone, $n = 1$), while cones of a given type had a homogeneous spectrum after replacement of their native chromophore(s) with A_1 retinal (◇, red cones, $n = 4$; △, blue cones, $n = 5$) (some results from Makino et al., 1990, 1991 have been included). (*B*) Spectral sensitivities of four UV cones showing the range in relative sensitivities to wavelengths >420 nm. For these cells, i_0 ranged from 420 to 1,200 photons·μm^{-2} at 366 nm, 3.8×10^4 to 6.2×10^5 photons·μm^{-2} at 480 nm, and 1.1×10^6 to 1.2×10^8 photons·μm^{-2} at 600 nm.

all hypsochromically shifted native spectra were well fit by a curve generated from a weighted sum of A_1 and A_2 retinal based pigments (see Methods). Furthermore, replacement of the cell's native chromophore(s) with exogenously applied A_1 chromophore shifted their spectral sensitivities to shorter wavelengths and produced homogeneous spectra for all red cones and for all blue cones. These spectra had characteristic A_1 pigment bandshapes (Ebrey and Honig, 1977; Mansfield, 1985; MacNichol, 1986; Baylor et al., 1987) (Fig. 3 *A*). Thus, whereas A_2 chromophore usually predominates (Harosi, 1975), the proportion of A_1 was as high as 70% in the cones of some retinas. Rods, sampled from different salamanders, contained up to 50% A_1 pigment. The chromophore mixture may be an early sign of metamorphosis, which includes a changeover from A_2 to A_1 (Harosi, 1976). Variability of this magnitude was reported in visual pigment extracts from retinas of individual larval axolotls, metamorphosed axolotls, and adult tiger salamanders (Ernst et al., 1978).

In contrast to the spectra of red cones, rods, and blue cones, which showed monotonic declines on their long-wavelength sides, the spectrum of the UV cone exhibited an unusual shoulder between 400 and 500 nm (Makino et al., 1991; Harosi, 1994) and a secondary maximum near 600 nm (Fig. 2). The possibility that this was the result of simultaneously recording from a UV cone, a blue cone, and a red cone, each in the same suction pipette, was ruled out by careful visual inspection of the cell before and after recording. In addition, whole-cell voltage clamp recordings of individual UV cones yielded similar results ($n = 3$). Measurements of flash sensitivity at long wavelengths were unchanged by the insertion of an additional UV-blocking filter in the optical path, ensuring that sensitivity at long wavelengths did not arise spuriously from leakage of some UV light through the interference filters. The spectral locations of the shoulder and the secondary maximum in the UV cone were more consistent with the presence, in addition to a UV-absorbing pigment opsin, of two additional opsins previously thought to be expressed only in blue and red cones, respectively.

In UV cones, i_0 ranged from 3.3×10^2 to 3.5×10^3 photons·μm^{-2} at 366 nm ($n = 11$), from 3.5×10^4 to 6.2×10^5 photons·μm^{-2} at 480 nm ($n = 11$), and from 1.1×10^6 to 1.2×10^8 photons·μm^{-2} at 600 nm (in those cells where sensitivity at 600 nm was measurable at two flash strengths, $n = 7$). After normalizing for the variation in sensitivity at the maximum, the amplitudes of the shoulder and the secondary maximum still varied considerably from cell to cell (Fig. 3 *B*). Sensitivity to 480 nm in some UV cones was tenfold higher than in others. At 600 nm, sensitivity was sometimes below the level of detection, indicating that sensitivity to this wavelength differed by more than a factor of 100. The lack of correlation between high sensitivity at 366, 480, or 600 nm suggests that the amount of each type of opsin not only varied but did so independently.

The small spectral shift of UV cones upon complete conversion from A_2 to A_1 pigment (Harosi, 1994) made it difficult to evaluate the chromophore proportions of the UV pigment in the cones of this study. Analysis was further complicated by the presence of the shoulder and secondary maximum in the spectrum. However, the other cone types as well as the rods contained chromophore mixtures, making it likely that both chromophores were also available to UV cones. Bleached UV cone opsin readily combines with exogenously applied A_1 (unpublished results), so it seems probable that native UV pigment was also present as a mixture.

Published July 1, 1996

Then the UV cone may contain as many as six visual pigments, formed from the combination of two chromophores and three opsins.

Partial Bleaching of UV Cone

Partial bleaching experiments (Dartnall, 1957) provided a further test for the presence of multiple opsins in the UV cone. Intense colored light was used to differentially alter the pigment content of a UV cone. A red bleaching light containing wavelengths >610 nm reduced the secondary maximum in the spectrum without affecting sensitivity at the peak or at the shoulder (n = 3) (Fig. 4 *A*). A bleaching light containing wavelengths >475 nm differentially reduced the sensitivity to wavelengths longer than 420 nm (n = 5) (Fig. 4 *B*). In one cell, bleaching with 366-nm light (~10 nm bandwidth) reduced sensitivity at 366 nm about twofold more than that at 500 nm. Thus, the three regions of the spectrum could be manipulated separately.

UV Pigment Bandwidth

The bandwidth at half-maximal sensitivity of the UV cone spectrum was 0.45 μm^{-1}, less than that of the blue cone, 0.57 μm^{-1}, and of the rod, 0.49 μm^{-1}, but slightly greater than that of the red cone, 0.40 μm^{-1} (Fig. 2). A similar trend has been described from microspectrophotometry, where the bandwidths of the salamander UV cone, blue cone, rod, and red cone were measured to be 0.50, 0.51, 0.49, and 0.41 μm^{-1}, respectively (Harosi, 1994). Narrow bandwidths have also been reported for UV cones of zebrafish (Robinson et al., 1993) and rainbow trout (Hawryshyn and Harosi, 1994). It may well be that narrow bandwidth is a universal property of UV-sensitive visual pigments (Harosi, 1994).

High Photosensitivity of UV Pigment

Intense flashes of light produce brief current transients in photoreceptors caused by charge movements that accompany pigment bleaching (Cone and Pak, 1971). The amplitude of this early receptor current (ERC) is proportional to the number of pigment molecules bleached (see, for example, Hodgkin and O'Bryan, 1977), so its decline with successive flashes was used to determine the pigment's photosensitivity, P, defined as the product of the molecular cross section for photon capture and the quantum efficiency of photoisomerization (Goodeve and Wood, 1938). The total amount of charge moved, R_0, ranged from 0.770 to 6.370 pC in the red cones and from 1.037 to 2.348 pC in the UV cones studied. P determined for red cone pigment (Fig. 5 *A*), (8.8 ± 1.2) × 10^{-9} μm^2·molecule^{-1} (mean ± SD, n = 13), was slightly larger than the value obtained previously, (7.2 ± 0.9) × 10^{-9} μm^2·molecule^{-1} (Makino et al., 1991), possibly because of a higher A_1

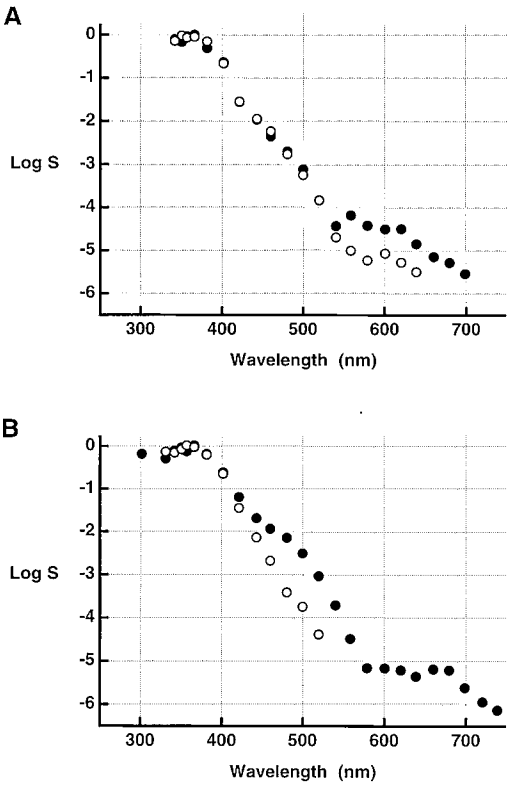

FIGURE 4. Changes in spectral sensitivity after partial bleaching of the UV cone. Filled symbols show the spectrum of the dark-adapted cone, and open symbols show the spectrum after intense irradiation. (*A*) Bleaching with light of ~84 $\mu W \cdot cm^{-2}$ containing wavelengths longer than 610 nm over a period of 5 min had little effect on i_0 at 366 nm, which only changed slightly from 1,250 photons·μm^{-2} to 1,640 photons·μm^{-2}. However, i_0 at 640 nm increased from 8.74 × 10^7 to 4.58 × 10^8 photons·μm^{-2}. (*B*) An 8-min exposure to light of ~110 $\mu W \cdot cm^{-2}$, containing wavelengths longer than 475 nm, increased i_0 from 780 photons·μm^{-2} to 9,410 photons·μm^{-2} at 366 nm but increased i_0 from 1.14 × 10^5 to 2.28 × 10^7 photons·μm^{-2} at 480 nm.

content in the cells of this study. P for the UV-sensitive pigment (UV pigment) (Fig. 5 *B*), was (12.4 ± 2.7) × 10^{-9} μm^2· molecule^{-1} (n = 6). This is smaller than the prior report of 44 × 10^{-9} μm^2·molecule^{-1} from a single determination (Makino et al., 1991). Nevertheless, P for the UV pigment of this study remained larger than that for red cone pigment ($p < 0.01$). Recalculation of P for the cell of the earlier study using the component analysis (see Methods) and the current UV cone spectral sensitivity yielded a value of 14 × 10^{-9} μm^2·molecule^{-1}.

DISCUSSION

Absorption Band Overlap

The absorption bandwidth of visual pigments decreases as its spectral maximum shifts to longer wavelength (Liebman and Entine, 1968; Smith and Pokorny, 1972;

Published July 1, 1996

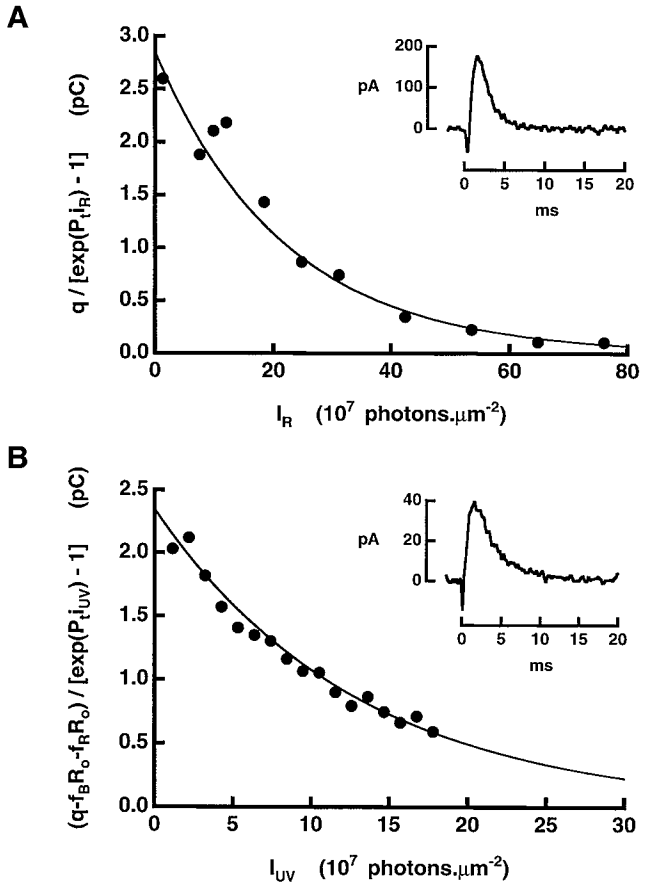

FIGURE 5. Cone pigment photosensitivity. ERC records were digitally filtered at 1 kHz. (*A*) Red cone pigment. The continuous line shows the exponential fit, with an R_0 of 2.850 pC and P_t of 4.6×10^{-9} μm^2·molecule^{-1}. (*Inset*) ERC elicited by a 575-nm flash. The effective flash strength was 6.4×10^7 photons·μm^{-2}. Presented after 49% of the cell's pigment had already been bleached, the flash bleached an additional 5%. (*B*) UV cone pigment. R_0 was 2.348 pC and P_t was 7.8×10^{-9} μm^2·molecule^{-1}. (*Inset*) ERC produced by a flash of white light (KV380 filter). The flash, of effective strength 1.1×10^7 photons·μm^{-2}, was delivered after 22% of the cell's pigment had already been bleached.

Ebrey and Honig, 1977). Theoretical calculations indicate that this will occur with greater delocalization of the chromophore's π electrons by opsin (Greenberg et al., 1975; Honig et al., 1976). Unexpectedly, spectra of UV-absorbing pigments introduced a reversal in this trend (Harosi, 1994; Hawryshyn and Harosi, 1994). One explanation might be that in visual pigment spectra, the effect of electron delocalization on bandwidth is overshadowed by a second effect, namely, the relative spectral locations of two adjacent absorption bands. In long wavelength–sensitive pigments, there is only a slight overlap of the main α band with the minor β band, a shorter wavelength absorption arising from the *cis* bond in the chromophore (Knowles and Dartnall, 1977*a*). In middle wavelength–sensitive pigments, the overlap may increase and cause an apparent broadening of the main absorption band (Harosi, 1976). Broadening reaches a maximum in short wavelength–sensitive pigments with peak sensitivity near 430 nm (Harosi, 1994). Further overlap of the two bands in UV pigments then narrows the bandwidth.

Besides affecting bandwidth, increasing overlap of the two bands should increase photosensitivity, assuming that the mechanisms of spectrally tuning the pigment do not also affect *P*. For additive absorptions, the relative sensitivities of the average red cone to 440 and 600 nm suggest that *P* for the UV pigment could be as much as 1.2× larger than that for the red pigment or 10.6×10^{-9} μm^2·molecule^{-1}. This is similar to the observed value, 12.4×10^{-9} μm^2·molecule^{-1}. Smaller differences in *P* between other visual pigments are also expected but have not yet been resolved (Liebman and Entine, 1968; Dartnall, 1972; Crescitelli and Liu, 1985; Crescitelli et al., 1985; Gupta and Williams, 1990; Makino et al., 1991; Jones et al., 1993, but see Okano et al., 1992).

Multiple Opsins in the UV Cones

The absorption spectrum of fly photoreceptors has three lobes. Flies possess an accessory pigment that absorbs UV light and then transfers the energy to a rhodopsin that exists in two photoreversible forms, whose maxima are ~90 nm apart (Kirschfeld et al., 1977). However, the long wavelength–absorbing form of fly rhodopsin does not elicit a measurable photoresponse (Hillman et al., 1983). Furthermore, partial bleaching experiments in salamander UV cones yielded results inconsistent with a sensitizing pigment mechanism.

Use of three chromophores could provide the basis for the salamander UV cone's spectrum, although only two chromophores, 11-*cis* retinal and 3-dehydro 11-*cis* retinal, have ever been observed to combine with vertebrate opsins naturally (Goldsmith, 1990; Crescitelli, 1991). If novel chromophores were present, they would also be expected to generate multiple pigments in red cones, blue cones, and rods. Since this was not observed, it seems more likely that three different opsins are present in the UV cone.

Thus, the salamander UV cone contains three functional opsins. From their spectral positions, two of the opsins may be identical to those present in red and blue cones, respectively. Apparently, the expression of one opsin in this cell type did not preclude that of others. This did not appear to be true of every type of salamander photoreceptor. In blue cones, for example, red pigment molecules were excluded to better than one part in a million, the limit of detection in this study. Some specificity of opsin expression in the UV cone was maintained, however. Whereas the red and blue cone opsins appeared to be present, rod opsin was

Published July 1, 1996

not. The relative amount of each opsin expressed and the relative chromophore proportions in the UV cone varied from cell to cell and may be subject to regulation.

Although physiological function has not yet been demonstrated, evidence has been accumulating to suggest that two visual pigments coexist in single photoreceptors of fish and rodent. This may result from the heterozygous expression of two polymorphic forms of an opsin (Archer et al., 1987; Archer and Lythgoe, 1990) or from a developmental shift in the type of opsin expressed (Shand et al., 1988; Wood and Partridge, 1993; Szel et al., 1994). Eel rods are capable of changing both chromophore and opsin type during maturation (Wood and Partridge, 1993). If multiple pigments in the salamander UV cone arise from a developmental shift in opsin expression, then two such shifts occur even before any outward signs of metamorphosis into the terrestrial stage. Changeover in opsin expression may serve as a slow but significant mechanism for shifting spectral sensitivity, whereas a switch in chromophore type may be faster and more important for fine-tuning. Alternatively, retinas of adult mouse, guinea pig, and rabbit contain a subpopulation of cones with two opsins (Rohlich et al., 1994), perhaps reflecting natural selection for cells with broad spectral sensitivity. More extreme evolutionary pressures may have resulted in the use of the two most spectrally disparate pigments known in salamander UV cones.

Recently, UV cones were identified in gecko microspectrophotometrically (Loew et al., 1996). In two of the three species examined, the UV cone spectrum had a maximum near 365 nm and a shoulder between 400 and 500 nm, possibly because of the coexistence of UV and blue pigment opsins in this cell type. Invertebrate photoreceptors that contain multiple pigments or a sensitizing pigment always have a UV-sensitive component (Menzel, 1979). The occurrence of three opsins in the salamander UV cone and putative two opsins in gecko suggests that this generality might extend to vertebrate UV photoreceptors.

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