

Relief of opsin desensitization and prolonged excitation of rod photoreceptors by 9-desmethylretinal

(rhodopsin/retinoids/bleaching adaptation/vision)

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ABSTRACT The 9-methyl group of 11-*cis*-retinal plays a crucial role in photoexcitation of the visual pigment rhodopsin. A hydrogen-substituted analogue, 11-*cis*-9-desmethylretinal, combines with opsin to form a pigment that produces abnormal photoproducts and diminished activation of the GTP-binding protein transducin *in vitro*. We have measured the formation of this analogue pigment in bleached salamander rods and determined the size and shape of its quantal response. In addition, we have characterized the influence of opsin and newly formed analogue pigment on the quantal response to native porphyropsin. We find that, as 11-*cis*-9-desmethylretinal combines with opsin in bleached rods, the amplitude of the quantal response from residual native pigment is elevated by ≈ 7.5 -fold to 0.15 ± 0.09 pA, a value close to the amplitude of the quantal response before bleach (0.31 ± 0.10 pA). When activated by light, the new analogue pigment produces a quantal response that is ≈ 30 -fold smaller and decays ≈ 5 times more slowly than that of native pigment in unbleached cells. We conclude that the 9-methyl group of retinal is not critical for conversion of opsin to its nondesensitizing state but that it is critical for the normal processes of activation and deactivation of metarhodopsin that give rise to the quantal response.

Photoisomerization of 11-*cis*-retinal (Fig. 1, structure 1) initiates an intramolecular rearrangement of rhodopsin that results in a catalytically active state of rhodopsin, R^* (1–5). Deactivation of R^* requires phosphorylation by rhodopsin kinase (6, 7) and the subsequent binding of arrestin (3, 7–9). In isolated photoreceptors, pigment activation and deactivation produce a discrete electrical response with a characteristic amplitude and time course (10, 11). In an examination of the steric interactions between the apoprotein opsin and its chromophore, Ganter *et al.* (12) reported that 11-*cis*-9-desmethylretinal (Fig. 1, structure 3) produced abnormal photoproducts and transducin activation that was 8% of the rhodopsin control. Here we examine the influence of the 9-methyl group of retinal on the amplitude and shape of the quantal response in isolated rods.

To provide access to the ligand binding pocket of opsin, the native chromophore (13) 11-*cis*-3,4-dehydroretinal (Fig. 1, structure 2) was removed by bleaching. Bleaching reduces the sensitivity of a cell by depleting the supply of native pigment and by reducing the amplitude of the quantal response from the residual pigment (14–17). In the absence of 11-*cis*-retinal, this desensitization persists indefinitely (15, 18) and is unresponsive to the addition of all-*trans*-retinal (15, 19) or its removal from opsin by hydroxylamine (20, 21). We refer to the persistent component of desensitization that results from response attenuation and does not require the

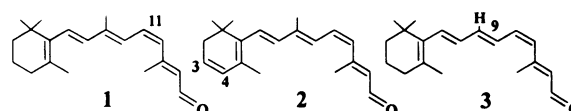


FIG. 1. Chromophores of the visual pigments considered in the experiments that follow: 1 (rhodopsin), 11-*cis*-retinal; 2 (porphyropsin), 11-*cis*-3,4-dehydroretinal; 3 (9-desmethyl rhodopsin), 11-*cis*-9-desmethylretinal.

presence of a retinal-containing photoproduct as opsin desensitization. Taken together with the loss of sensitivity resulting from pigment depletion, the total loss of sensitivity is commonly referred to as bleaching adaptation (14, 17). Retinal analogues may restore sensitivity by increasing the quantity of photoexcitable pigment, by neutralizing the effects of free opsin, or by a combination of these activities.

Photochemically inactive analogues of retinal—e.g., 11-*cis*-locked retinal (15)—and fragments of retinal as small as β -ionone (22) have been shown to neutralize the effects of free opsin. In the course of these experiments, we have examined the role of the 9-methyl group in restoring opsin to its inactive dark-adapted state upon pigment formation. We find from our analysis of pigment content and quantal responses that formation of 9-desmethyl rhodopsin effectively neutralizes free opsin but that, when activated, the 9-desmethyl pigment produces a quantal response that decays more slowly and is ≈ 30 -fold smaller than that of native pigment.

A preliminary report of these findings has appeared in abstract form (23).

MATERIALS AND METHODS

11-*cis*-9-Desmethylretinal was prepared by condensation of *trans*-5-(2',6',6'-trimethylcyclohex-1'-enyl)penta-2,4-dienal with the anion derived from bis(trifluoroethyl)-2-methyl-3-cyano-2-propenyl phosphonate followed by reduction of the resulting 11-*cis*- and 11-*trans*-9-desmethylretinonitriles with diisobutylaluminum hydride (24, 25). The 11-*cis* isomer of 9-desmethylretinal was separated from the *trans* isomer by HPLC (μ -porasil; hexane/ethyl acetate solvents) and characterized by absorption and NMR spectroscopy. The analogue was delivered to isolated rods in phosphatidylcholine vesicles in physiological saline as described (14, 15).

Unpolarized monochromatic light stimuli were provided by an optical bench containing a tungsten/halogen lamp, Oriel (Stamford, CT) interference filters (10-nm bandpass), neutral density wedges, and fast electromagnetic shutters (Uniblitz, Rochester, NY) and were calibrated with a PIN photodiode radiometer (United Detector Technology, Santa Monica, CA) as described (15).

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Light-evoked currents were recorded from the inner segment of rods of the tiger salamander *Ambystoma tigrinum* by means of a suction electrode connected to a current-to-voltage converter (List Electronics, Darmstadt, F.R.G., L/M-EPC7 patch clamp amplifier) (14, 15). The responses were filtered at 5 Hz with an eight-pole Bessel filter [Frequency Devices (Haverhill, MA) model 902] and digitized on-line by a 12-bit A/D converter [Scientific Solutions (Solon, OH) Labmaster Board].

Flash sensitivity, S_F , is defined for conditions near threshold as the ratio of the incremental response to the increment in light intensity (26). This quantity was estimated either directly from the response amplitude and intensity of dim flashes or from the slope of the low-intensity asymptote of stimulus-response functions (15, 27). The amplitude of the single photon response can be estimated as originally described by Baylor and coworkers (11). From Campbell's theorem, the ratio of the light-induced increase in the variance, σ^2 , to the mean amplitude, μ , of the dim flash response provides a raw estimate of the amplitude of the quantal response (11). A refined estimate, A , of the net quantal response of the cell in pA was obtained from Eq. 1. In this equation, the raw estimate given by the first term (σ^2/μ) is corrected upward by $\approx 16\%$ in these experiments by the second term, which compensates for the loss of variance arising from response saturation (28, 29). The value of the exponent (0.5) was obtained empirically from the analysis of simulated responses having an average size ($\approx 25\%$ of R_{\max}) and steepness of saturation characteristic of the data in these experiments. The estimate is also corrected upward by $\approx 22\%$ in these experiments by a third term, R_m/R_0 , which adjusts for the collection efficiency of the pipette (30, 31). In this equation, R_s is the amplitude of the response at saturation, R_m is the maximum current (55 pA) of a typical cell under voltage clamp (30, 31), and R_0 is the amplitude of the response at saturation for the unbleached cell at the start of an experiment. Note that after bleach, R_s is considerably smaller than R_0

$$A = (\sigma^2/\mu)[R_s/(R_s - \mu)]^{0.5}(R_m/R_0). \quad [1]$$

In these experiments, μ was estimated from the peak of the ensemble average of the dim flash response. The variance, σ^2 , was estimated by a least-squares fit of the square of the average response waveform to the ensemble increase in variance. Before calculating the ensemble waveform of the variance, the preflash baseline of each individual response was established by fitting the average response to each individual response after removal of any linear trend (drift) to minimize distortion. As any drift in the average response size can inflate the estimate of variance, the variance of the records was computed for blocks of 10 records and then pooled (32).

For measurements of the optical density of rods, a beam containing unpolarized monochromatic light (5-nm bandwidth) was generated with an Oriol 1/8-m double monochromator and used to focus images of sample and reference slits ($3 \times 20 \mu\text{m}$) in the plane of the cell for spectroscopic measurements. The transverse optical density of a cell was determined by comparing photon counts from the sample beam with the reference beam by means of an imaging photon detector made by Instrument Technology Limited (East Sussex, U.K.; model IPD). The sample beam did not bleach appreciable amounts of pigment, as no loss of sensitivity could be detected after the spectral measurements.

The number of pigment molecules in a rod was estimated from the cell dimensions and the transverse optical density as described by Hárosi (13) and assuming a molar extinction coefficient of 29,400 liters \cdot mol $^{-1}\cdot$ cm $^{-1}$ for the native porphyropsin (11-*cis*-3,4-dehydroretinal; $\lambda_{\max} = 523 \text{ nm}$) and 41,000

liters \cdot mol $^{-1}\cdot$ cm $^{-1}$ for 9-desmethyl rhodopsin ($\lambda_{\max} = 465\text{--}470 \text{ nm}$) (33) and a dichroic ratio of 3.78 for porphyropsin and 4.55 for the analogue pigment (see ref. 13). In these experiments, we have simplified our calculations by ignoring the minor fraction of rhodopsin (11-*cis*-retinal; $\lambda_{\max} = 498 \text{ nm}$), which occurs in salamander rods (13). An optimal estimate of transverse optical density was obtained by cross-correlation of a visual pigment template with the raw optical density values.

The number of photoactivated molecules, R^* , expected from a dim test flash was estimated from Eq. 2 (see ref. 34) given that

$$R^* = R_0[1 - \exp[-\alpha(\lambda)\gamma\delta it]]. \quad [2]$$

R_0 is the initial number of molecules; $\alpha(\lambda)$ is the molecular extinction in μm^2 ; γ is the quantum efficiency of excitation; λ is the wavelength in nm; δ is the fractional efficiency of unpolarized light; i is the light intensity in photons $\mu\text{m}^{-2}\cdot\text{sec}^{-1}$; and t is the exposure time in sec. Note that the quantum efficiency γ was assumed to be 0.65 for both pigments and the fractional efficiency δ was 0.63 for porphyropsin and was assumed to be 0.61 for analogue pigment (13). Cells were bleached for 8 sec with a 520-nm bleaching beam at an intensity of $1.36 \pm 0.13 \times 10^8$ photons $\cdot\mu\text{m}^{-2}\cdot\text{sec}^{-1}$.

RESULTS

To examine the activity of 11-*cis*-9-desmethylretinal, the native visual pigment of an isolated salamander rod was bleached away, and the 9-desmethyl analogue was applied from lipid vesicles. As shown in Fig. 2, bleaching accelerated the response kinetics and reduced the maximum response amplitude. Of five bleached cells, all exhibited accelerated response kinetics and a reduction of the maximum response amplitude to $46\% \pm 14\%$ (mean \pm SD) of the prebleach value of $45 \pm 7 \text{ pA}$. Sensitivity was reduced by 2.5 ± 0.1 log units.

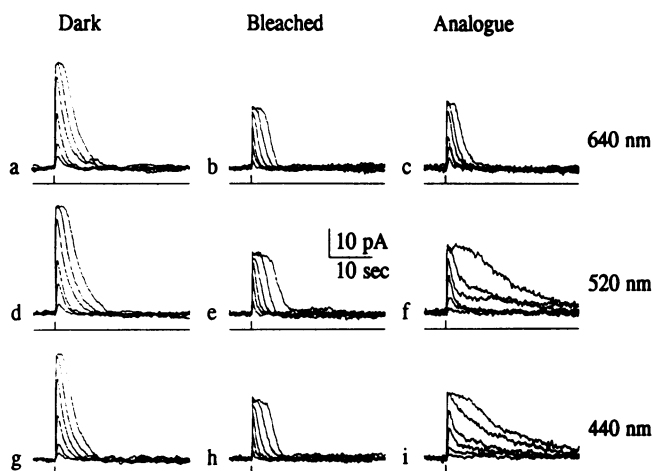


FIG. 2. Changes in response waveforms induced by bleaching and by application of 11-*cis*-9-desmethylretinal. Series of test flashes (20 msec) ascending in brightness in 0.5-log-unit steps were applied to a rod until the response reached saturation for three wavelengths (rows as indicated) and three experimental conditions: dark adapted (column 1), bleached (column 2), and resensitized with 9-desmethylretinal (column 3). Bleaching results in a reduction of maximum response amplitude and an acceleration of the response kinetics. Addition of 9-desmethylretinal results in a partial reversal of both these effects. In addition, the response becomes abnormally prolonged in a wavelength-dependent manner as shown in column 3. For the responses to 20-msec flashes illustrated above, the maximum flash intensity in units of photons/ μm^2 was as follows: a, 6.34×10^3 ; b, 2.01×10^6 ; c, 2.01×10^5 ; d, 2.71×10^2 ; e, 8.57×10^4 ; f, 8.57×10^3 ; g, 2.76×10^2 ; h, 8.72×10^4 ; i, 2.76×10^3 .

Of this loss, 1.3 log units can be attributed to a $95\% \pm 3\%$ depletion of pigment as measured spectroscopically. The remaining loss of ≈ 1 log unit results from a reduction in the quantal response amplitude as confirmed by variance measurements. In accord with previous observations (11), there was no evidence of wavelength dependence among response shapes before or after bleaching of native pigment.

Application of 11-*cis*-9-desmethylretinal to bleached rods elevated sensitivity to 520-nm test flashes by 1.1 ± 0.4 log units, partially reversed the acceleration of response kinetics induced by bleaching, and restored the maximum response amplitude to $67\% \pm 6\%$ of the original amplitude. After application of the analogue, the shape of the response waveform became dependent on the wavelength of stimulation as shown in Fig. 2 *c*, *f*, and *i*. As we consider in detail below, this wavelength dependence of the response shape appears to result from the mixed activities of residual native pigment, which produces normal responses with maximal sensitivity near 520 nm, and new analogue pigment, which produces small but prolonged responses with maximal sensitivity near 470 nm and negligible activity at 640 nm relative to that of the residual native pigment. It is possible to separate the increase in sensitivity due to relief of opsin desensitization from the increase in sensitivity resulting from analogue pigment formation by selective stimulation of analogue or residual native pigments at different wavelengths and determination of the quantal response amplitude from the mean and variance of the responses to dim flashes as shown in Figs. 3 and 4.

The quantal response of native pigment molecules can be determined under the conditions shown in Fig. 3 for porphyropsin molecules in the presence of (i) other native pigment molecules before bleach, (ii) opsin after bleach, or (iii) 9-desmethyl rhodopsin after application of 11-*cis*-9-desmethylretinal. As shown by the average response shape in Fig. 3*a*, the response of an unbleached and dark-adapted cell increases to a peak and then decays. A semilogarithmic plot of the response shown in Fig. 3*b* reveals the decay to be

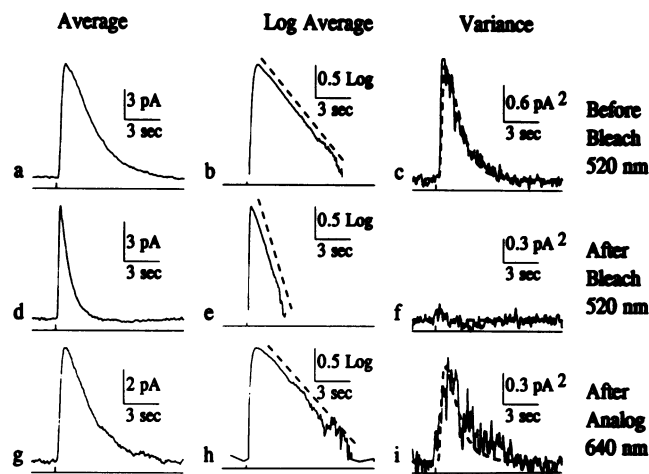


FIG. 3. Analysis of the kinetics and variance of dim flash responses of native pigment under conditions of dark adaptation before bleach (top row), after bleach (middle row), and after resensitization with 11-*cis*-9-desmethylretinal (bottom row). Column 1 (*a*, *d*, and *g*) contains the waveforms of the average dim flash responses and shows the acceleration of response kinetics induced by bleaching and the partial recovery observed after application of 9-desmethylretinal for averages of 50 responses. In column 2 (*b*, *e*, and *h*), the logarithms of the normalized responses are plotted together with regression lines fitted to the falling phase of the responses but offset for clarity (dashed line). Column 3 (*c*, *f*, and *i*) shows the light-induced increase in the variance of the responses. The expected waveform and estimated amplitude of the light-induced increase in variance is given by the scaled square of the response waveform (dashed curve) as described.

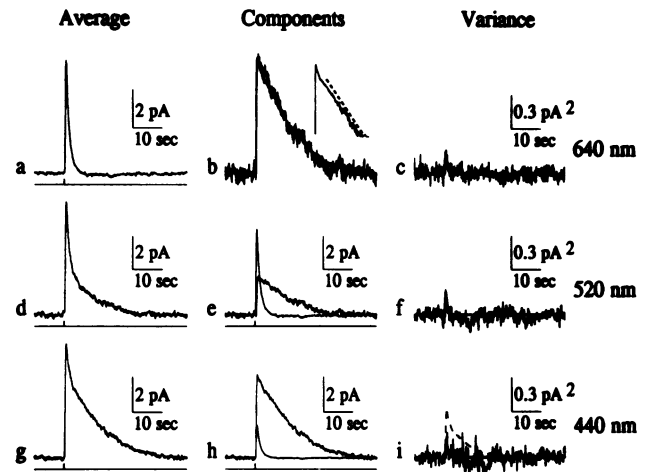


FIG. 4. Analysis of the wavelength-dependent components of the dim flash response obtained from averages of 50 flashes. The average dim flash response obtained with 640-nm flashes is shown in *a*. In the average responses illustrated in *d* at 520 nm and in *g* at 440 nm, a slowly decaying component of the response becomes progressively more prominent on the average. A semilogarithmic plot of the response in *g* (Inset in *b*) reveals a slow component of decay that is linear and hence approximately exponential in character with a time constant of 11.6 sec. From the semilogarithmic plots, which exhibited prominent shallow and linear slopes in *d* and *g*, the average responses at these wavelengths were decomposed into the sums of fast (640 type; *a*) and slow responses as indicated in *e* and *h*, respectively. The slow components were then normalized and plotted in *b*, where they appear to scale linearly. The light-induced increase in the variance of these records is plotted in column 3. Smooth solid line indicates estimated variance obtained by fitting the square of the average response to the waveform of the light-induced increase in variance. Dashed line in *i* represents the expected sum of the squared waveforms of the fast and slow components in *h* under a hypothesis described in the text.

approximately exponential with a time constant of 2.0 sec for this cell and of 2.2 ± 0.4 sec overall ($n = 5$). The light-induced increase in variance for the dark-adapted cell is shown in Fig. 3*c*. From Eq. 1, we estimate the quantal response amplitude of this cell to be 0.23 pA and 0.31 ± 0.10 pA of all five cells under conditions of dark adaptation before bleach.

Bleaching reduces the time to peak of the response at 520 nm and accelerates the falling phase as shown in Fig. 3*d* and *e*. As shown in Fig. 3*e*, the response decays with an apparent first-order time constant of 0.74 sec for this cell and of 0.61 ± 0.12 sec for all five cells after bleach. In addition, the variance decreases and yields an estimate of the quantal response amplitude of 0.02 pA for the cell illustrated in Fig. 3*f* and of 0.02 ± 0.01 pA for all five cells, an ≈ 10 -fold drop in quantal response amplitude.

After addition of 11-*cis*-9-desmethylretinal, the response to residual native pigment molecules can be isolated with 640-nm test flashes. For these responses, the time constant of decay lengthened to 2.2 sec for the cell illustrated in Fig. 3*e* and to 1.65 ± 0.60 sec ($n = 5$) overall. From the variance of the response to 640-nm flashes, we estimate that the quantal response amplitude for residual porphyropsin grew to 0.19 pA for the cell illustrated in Fig. 3*i* and to 0.15 ± 0.10 pA ($n = 5$) overall, an average 7.5-fold increase in quantal response amplitude, which reverses most of the ≈ 10 -fold loss of quantal response amplitude that occurred on bleaching.

After addition of analogue, the dim flash response waveform became wavelength dependent as shown in Fig. 4 *a*, *d*, and *g*. The average responses in Fig. 4 *d* and *g* show evidence of an additional slowly decaying component in the response at shorter wavelengths. A plot of the logarithm of the response illustrated in Fig. 4*g* is shown in Fig. 4*b* (Inset),

which reveals a prominent linear slope in the latter part of the log response. The slope of the linear component was obtained by a least-squares fit shown by the offset dashed line, which corresponds to an exponential time constant of 11.6 sec. By extrapolating the slope of the slow component back in time to the onset of the flash, it was possible to estimate the amplitude of the fast component (640-nm type; Fig. 4*a*). The scaled waveform of the fast component was subtracted from the average to isolate the slow components as shown in Fig. 4*e* and *h*. When normalized as shown in Fig. 4*b*, the slowly decaying components illustrated in Fig. 4*e* and *h* have the same shape, as would be expected for the linear addition of the two components. In these experiments, the time constant for decay of the slow component was 11.5 ± 1.1 sec, ≈ 7 times longer than the comparable decay constant for the response at 640 nm and ≈ 5 times longer than before bleach.

The light-induced increase in variance is shown in Fig. 4*c*, *f*, and *i*. Note that a clear increase in variance is associated with the fast component of the response but that no increase in variance associated with the slow component is evident above the noise in Fig. 4*f* or *i*. The dashed curve in Fig. 4*i* illustrates the total light-induced increase in variance that would be expected if the quantal response of the slow component had the same amplitude as that of the fast component, which is represented by the solid curve.

Fig. 5 provides a summary of the sensitivity changes induced by bleaching and resensitization with 11-*cis*-9-desmethylretinal and an estimate of the quantal response size for the 9-desmethyl pigment, which could not be obtained from the variance measurements. Before bleach, the absorbance spectrum of the native 520-nm pigment in the cell was measured, smoothed, and plotted on normalized coordinates (solid circles, top right spectrum). The data were fitted with a porphyropsin pigment template 1 (solid line; G. J. Jones, personal communication), which peaks at 520 nm. Control measurements of the spectral sensitivity of six unbleached cells confirm the spectrum of the predominant pigment, porphyropsin (open squares). After bleach, the flash sensitivity of the cell fell 2.4 log units to the level of sensitivity indicated by the open triangles and solid pigment template 2. Two independent estimates were obtained to determine the portion of this loss of sensitivity that could be expected from pigment depletion. First pigment depletion was estimated spectroscopically at 1.6 log units as described and the result is plotted as a dashed line template 3. The relative number of native *R**s estimated from the mean and variance of the dim flash response after bleaching was then compared to the number of *R**s expected from a flash of that intensity given the prebleach pigment content, and the results are plotted as solid squares. This independent measurement confirms the spectroscopic estimate of ≈ 1.6 log units of sensitivity loss attributable to pigment depletion.

After the spectroscopic and sensitivity measurements following bleach, 11-*cis*-9-desmethylretinal was added and the formation of new analogue pigment was observed (solid circles, top left spectrum). A rhodopsin visual pigment template 5 with a peak at 470 nm shows that the new analogue pigment is well characterized by this template. From the final optical density of 0.124, we calculate that the analogue consumed $\approx 95\%$ of the available opsin in this cell.

After conversion of opsin to analogue pigment, the flash sensitivity of the fast component of the response increased by 0.6 log unit to the level indicated by the open diamonds and the solid line template 4. This level of sensitivity is very near the level expected for the residual native pigment (template 3). The proximity of the final sensitivity level for the fast component of the response to that predicted by pigment depletion confirms the quantal response measurements, which indicate that formation of analogue pigment substan-

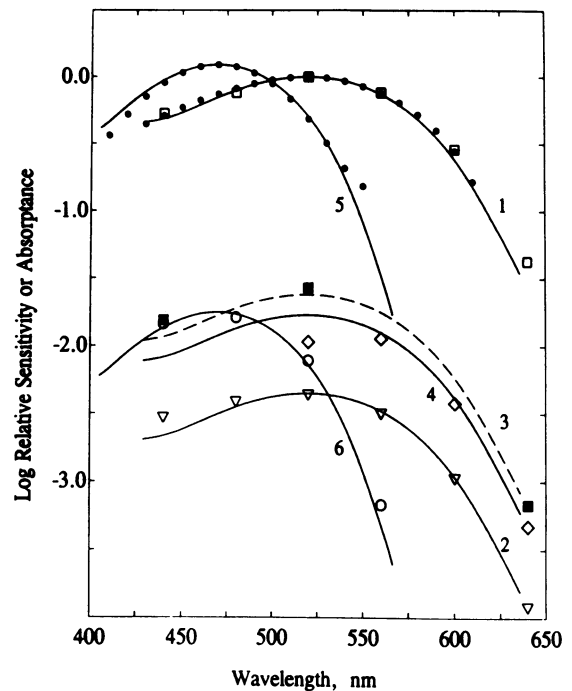


FIG. 5. Measurements of absorbance and spectral sensitivity. The absorbance spectra (solid circles) of the native pigment spectrum before bleach and the new 9-desmethylretinal analogue pigment are shown together with fitted pigment templates (1 and 5), which peak at 520 and 470 nm, respectively. The absorbance spectrum of the native pigment is normalized at 520 nm. The absorbance of the 9-desmethyl pigment is normalized to the peak of the absorbance of the native pigment. The spectral sensitivity of the cell was measured before bleach and normalized to the peak at 520 nm (open squares). After bleach, the spectral sensitivity was again measured and found to have fallen by 2.4 log units (open triangles and template 2). A spectroscopic estimate of the level of sensitivity expected after simple pigment depletion is plotted as the dashed native pigment template 3. An independent estimate of the relative amount of residual native pigment obtained from variance measurements after bleach is shown as solid squares. After addition of 11-*cis*-9-desmethylretinal, the flash sensitivity of the residual native pigment was estimated from the mean amplitude and light intensity of the fast component of dim flash responses and plotted relative to the initial sensitivity as the open diamonds and solid native pigment template 4, which lie close to the level predicted from pigment depletion alone (template 3). Finally, the flash sensitivity of the slow component of the dim flash responses is plotted relative to the initial sensitivity as the open circles, which have been fitted with the 9-desmethyl rhodopsin absorbance template 6.

tially removes the desensitization of the cell imposed by opsin.

We next plotted the flash sensitivity of the slow component of the responses (open circles) and fitted the analogue pigment template 6 to the data. From this plot, one can see that the sensitivity of the new analogue pigment is ≈ 1.7 log units lower (1.5 ± 0.2 ; $n = 5$) than would be expected from a fully functional pigment having the same density. This sensitivity measurement indicates either that the quantum efficiency of bleaching is much less than that of a normal pigment or that the quantal response amplitude is ≈ 30 -fold smaller.

DISCUSSION

After bleach, 11-*cis*-retinal, supplied by the pigment epithelium, enters the binding pocket of opsin and forms a Schiff base attachment to a lysine in the seventh transmembrane helix (5). In doing so, it induces a conformational change in the protein, which switches off opsin desensitization and

establishes the absorption maximum characteristic of rhodopsin. Substitution of an 11-*cis*-locked retinal produces both the characteristic opsin shift and relief of opsin desensitization without forming photoactivatable pigment (15). Substitution of a hydrogen atom for the methyl group at the 9 position results in a smaller opsin shift than normal (33). However, these experiments show that the 9-methyl group does not appear to be critical for the interaction that relieves opsin desensitization. As shown in Fig. 5, 9-desmethylretinal permits an increase in sensitivity for residual native pigment, which is close to the level predicted by pigment depletion alone after opsin has been replaced by analogue pigment.

In addition to relieving opsin desensitization, 11-*cis*-9-desmethylretinal forms a photoactive pigment. The optical density of the new pigment indicates that virtually all of the available opsin is consumed. However, as shown in Fig. 5, the flash sensitivity of the slow component of the response is 1.7 log units below that predicted for a normal pigment. This suggests that either the response to an individual analogue pigment molecule is much smaller than normal or that the quantum efficiency of excitation is much less than the value of 0.65 characteristic of other pigments (13). As shown in Fig. 4, the absence of a detectable increase in the variance associated with the slow component of the response suggests that it is response amplitude rather than quantum efficiency that is diminished.

In addition to a diminished amplitude, the quantal response for this analogue pigment appears to have a lifetime >7 times longer than that of residual native pigment. Although the quantal response amplitude is \approx 30-fold smaller than normal, the integral of this prolonged response yields a net response that is \approx 17% of the dark-adapted response, a value in rough agreement with that of 8% reported by Ganter and coworkers in their biochemical preparation (12).

One possible explanation for the small and prolonged response of the 9-desmethyl pigment is that the conformational changes normally induced in the protein on formation of metarhodopsin are altered or incomplete with the result that the metarhodopsin form of the pigment is not fully recognized by either transducin or rhodopsin kinase, which compete for the same binding site (35, 36). Under this hypothesis, phosphorylation of the protein is expected to be sluggish and/or incomplete. An initial study confirms a reduced phosphorylation of the 9-desmethyl pigment (37).

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