Dark Adaptation of Rod Photoreceptors in Normal Subjects, and in Patients with Stargardt Disease and an ABCA4 Mutation

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PURPOSE. Psychophysical and electroretinographic (ERG) studies indicate that patients with Stargardt disease exhibit abnormally slow rod dark adaptation after illumination that bleaches a substantial fraction of rhodopsin. However, relatively little information is available concerning rod recovery in this disease after weaker adapting (i.e., conditioning) light. With the use of a paired-flash ERG method, properties of the derived rod response to a low-bleach (<1%) but rod-saturating conditioning flash were investigated in seven normal subjects and in five Stargardt patients with identified sequence variations in the ABCA4 gene.

METHODS. In the first of two experiments, the interval between a fixed conditioning flash (67 or 670 scotopic cd s m⁻²) and a bright probe flash of fixed strength was varied to determine the falling-phase kinetics of the derived rod response to the conditioning flash. In the second, the instantaneous amplitude-intensity function for the rod response at an intermediate stage of recovery from the conditioning flash was determined by presenting a test flash of various strengths at a fixed time after the conditioning flash, and a probe flash at 200 ms after the test flash.

RESULTS. The maximum peak amplitude of the dark-adapted, rod-mediated a-wave determined in Stargardt patients (211 ± 87 μV) was on average lower than that determined in normal subjects (325 ± 91 μV; P = 0.06). The derived rod response to the 670 scotopic cd s m⁻² conditioning flash determined in normal subjects and Stargardt patients exhibited a biphasic recovery, and the kinetics of the early stage of recovery were similar in the two subject groups. For both normal subjects and patients, normalized amplitude-intensity functions describing the dark-adapted derived rod response exhibited half-saturation at approximately 1.5 log scotopic troland sec⁻¹. In both groups, the normalized amplitude-intensity function determined at approximately 2 seconds after the 67 scotopic cd s m⁻² conditioning flash and at approximately 9 seconds after the 670 scotopic cd s m⁻² conditioning flash exhibited an average desensitization (i.e., an increase of test flash strength at half-saturation) of approximately 0.5 to 0.6 log unit relative to that determined under dark-adapted conditions.

CONCLUSIONS. The results indicate that, despite a reduction in the average dark-adapted maximum a-wave amplitude in the Stargardt/ABCA4 patients, the early-stage recovery kinetics of the derived rod response to a low-bleaching conditioning flash as well as the lingering rod desensitization produced by such a flash are similar to those determined in normal subjects. (Invest Ophthalmol Vis Sci. 2004;45:2447–2456) DOI:10.1167/iovs.03-1178

Stargardt disease is characterized by progressive loss in central vision and bilateral atrophic-appearing macular changes surrounded by yellowish-white fundus lesions at the level of the retinal pigment epithelium (RPE).1–3 Recent studies have shown that Stargardt disease is caused by sequence variations in the gene encoding ABCA4 (previously known as ABCR), a retina-specific protein expressed in rod and cone photoreceptors.4,5 The ABCA4 gene product is an active transporter that in rods facilitates the movement of all-trans retinal, the retinoid product of rhodopsin bleaching, from the lumen of the rod outer segment disc to the rod cytosol.6–10 This translocation and the subsequent enzymatic reduction of all-trans retinal terminate its ability to combine with opsin, and thus promote the shut-off of excitation in the phototransduction cascade1,11,12 (for reviews, see Refs. 13, 14). Consistent with this action of the ABCA4 transporter, many patients with Stargardt disease and ABCA4-associated cone–rod dystrophy require an abnormally long period to dark adapt fully, after adapting illumination that bleaches a major portion of their rhodopsin.1,15–19 Similarly, the abcr knockout mouse, which lacks the ABCA4 gene product, exhibits abnormally slow recovery of the electroretinographic (ERG) a-wave after substantial rhodopsin bleaching.20

A question of interest raised by the available data is whether the prolongation of dark adaptation in ABCA4 Stargardt patients occurs only with bleachings that generate a substantial amount of all-trans retinal photoprodut. The present study was undertaken to compare rod flash sensitivity after weak bleaching illumination in normal subjects and in Stargardt patients with identified ABCA4 mutations, using a paired-flash electroretinographic (ERG) method that allows determination of the instantaneous amplitude-intensity function at defined times after a conditioning flash.21–22 Preliminary results have been reported (Pepperberg DR, et al. IOVS 2001;12:ARVO Abstract 4203).

METHODS

All procedures were in accordance with institutional policies and with the principles embodied in the Declaration of Helsinki. The experi-

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at stages 1 and 2, respectively, as previously described. Two subgroups of patients would have been classified with localized fundus flecks in the parafoveal region; three additional patients with more diffuse flecks. These two subgroups of patients would have been classified as having disease at stages 1 and 2, respectively, as previously described.2

Electroretinography

Before each experiment, the pupil of the eye to be tested was dilated with 2.5% phenylephrine HCl and 1% tropicamide. An opaque patch was placed over the eye for 45 to 60 minutes to allow for full dark adaptation. Under dim red light, the patch was removed and the corneal surface anesthetized with 0.5% proparacaine HCl. ERGs were recorded with the use of a bipolar electrode (GoldLens; Diagnosys, LLC, Littleton, MA) placed on the corneal surface. The corneal surface was lubricated with several drops of methylcellulose (Celluvisc; Allergan, Irvine, CA) placed on the electrode surface immediately before its installation. A ground electrode (Model E34D; Grass-Telefactor, West Warwick, RI) was clipped to the ipsilateral ear. The subject was seated at a Ganzfeld dome in a dark room and asked to focus on a small red fixation light positioned axially in the dome.

A short-wavelength (blue) flash that preferentially stimulated rod photoreceptors (Watten 47B filter, \( \lambda_{\text{max}} = 449 \) nm; Eastman Kodak Co., Rochester, NY) was used as a test flash. The test flash was provided by a Novatron flash unit (model 2105-C flash unit and 1000 VR power pack; Novatron Inc., Dallas, TX), a short-wavelength flash (Watten 47B filter; Eastman Kodak) that ranged in strength from 302 to 2400 sc cd s m\(^{-2}\). A long-wavelength (red) flash (\( \lambda_{\text{cut-off}} = 605 \) nm; Watten 26, Eastman Kodak) of intensity photopically matched to the short-wavelength probe was used for determination of the cone contribution to the nominal probe flash response (described later). A second Novatron unit provided the bright, short-wavelength conditioning flash. Light from both the two Novatron flash lamps and the Grass flash lamp passed through heat filters (catalog no. 66.2450; Rolyn Optics Co., Covina, CA) and was attenuated with neutral density filters (Wratten; Saunders Group, Rochester, NY). Flash strengths were calibrated using an integrating photometer (model 1700 with a model SED033 detector, radiance barrel and ZCIE scotopic filter; International Light, Newburyport, MA). Strengths of the conditioning flashes were 67 and 670 sc cd s m\(^{-2}\). Pupil diameters determined for the seven normal subjects tested were in the range of approximately 7.5 to 10 mm, and those for the five Stargardt ABCA4 patients ranged from approximately 7.5 to 9 mm. Determinations of pupil diameter typically were rounded to the nearest millimeter. The conversion of flash luminance \( L \) (in sc cd s m\(^{-2}\)) to

Using an Eastman Kodak (2105-C) flash unit, 1000 VR power pack, and Watten 47B filter (Eastman Kodak) of intensity photopically matched to the short-wavelength probe was used for determination of the cone contribution to the test flash response (described later). A second Novatron unit provided the bright, short-wavelength conditioning flash. Light from both the two Novatron flash lamps and the Grass flash lamp passed through heat filters (catalog no. 66.2450; Rolyn Optics Co., Covina, CA) and was attenuated with neutral density filters (Wratten; Saunders Group, Rochester, NY). Flash strengths were calibrated using an integrating photometer (model 1700 with a model SED033 detector, radiance barrel and ZCIE scotopic filter; International Light, Newburyport, MA). Strengths of the conditioning flashes were 67 and 670 sc cd s m\(^{-2}\). Pupil diameters determined for the seven normal subjects tested were in the range of approximately 7.5 to 10 mm, and those for the five Stargardt ABCA4 patients ranged from approximately 7.5 to 9 mm. Determinations of pupil diameter typically were rounded to the nearest millimeter. The conversion of flash luminance \( L \) (in sc cd s m\(^{-2}\)) to

Table 1. Description of Subjects

<table>
<thead>
<tr>
<th>Subject Number</th>
<th>Age</th>
<th>Sex</th>
<th>ABCA4 Variation</th>
<th>Max Peak a-Wave Amplitude (( \mu V ))†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>101</td>
<td>55</td>
<td>M</td>
<td>—</td>
<td>−243</td>
</tr>
<tr>
<td>102</td>
<td>37</td>
<td>F</td>
<td>—</td>
<td>−410</td>
</tr>
<tr>
<td>103</td>
<td>26</td>
<td>M</td>
<td>—</td>
<td>−188</td>
</tr>
<tr>
<td>104</td>
<td>23</td>
<td>F</td>
<td>—</td>
<td>−597</td>
</tr>
<tr>
<td>105</td>
<td>56</td>
<td>F</td>
<td>—</td>
<td>−268</td>
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<tr>
<td>111</td>
<td>29</td>
<td>F</td>
<td>—</td>
<td>−362</td>
</tr>
<tr>
<td>112</td>
<td>23</td>
<td>M</td>
<td>—</td>
<td>−410</td>
</tr>
<tr>
<td>106</td>
<td>50</td>
<td>F</td>
<td>val849ala, arg21107his</td>
<td>−325 ± 91</td>
</tr>
<tr>
<td>107</td>
<td>41</td>
<td>M</td>
<td>gly1961glu, arg2077trp</td>
<td>−201</td>
</tr>
<tr>
<td>108</td>
<td>22</td>
<td>M</td>
<td>ala60val, 1 bp ins codon 1513</td>
<td>−306</td>
</tr>
<tr>
<td>109</td>
<td>34</td>
<td>M</td>
<td>leu541pro/ala1038val,‡ gly1961glu</td>
<td>−82</td>
</tr>
<tr>
<td>110</td>
<td>51</td>
<td>M</td>
<td>gly1961glu</td>
<td>−191</td>
</tr>
</tbody>
</table>

* Age on the date of determination of the a-wave result shown in the right-hand column.
† Maximum peak amplitude of the dark-adapted a-wave response obtained with the brightest investigated test flash (955 or 2400 sc cd s m\(^{-2}\)). Entry at the bottom of each section identifies the mean ± SD of determinations for the indicated group of subjects.
‡ leu541pro and ala1038val are commonly found together on the same allele.
amplitude at time $t_{\text{det}}$. $A(t)$, the amplitude of the derived rod response to the conditioning flash at time $t$, was then obtained from the relation

$$A(t) = A_{\text{rod}}(t) - A_0(t) \quad (1a)$$

$$t = t_{\text{probe}} + t_{\text{det}} \quad (1b)$$

where $A_{\text{rod}}(t)$ is the amplitude of the rod-isolated probe response determined in the paired-flash trial, and where $A_0(t)$ is the rod-isolated probe response amplitude determined under dark-adapted conditions—that is, in the absence of recent presentation of the conditioning flash (probe-alone response).28,29 As $t_{\text{probe}}$ in all experiments greatly exceeded $t_{\text{det}}$ (7.0 - 8.4 ms), time $t$ in all of the reported data obtained in the type 1 experiments refers to the conditioning-probe interval $t_{\text{probe}}$.

Experiment 2 (lower part of Fig. 1) involved three flashes within each of a series of trials: a fixed, bright conditioning flash presented at time zero; a test flash of variable intensity presented at a fixed later time (conditioning-test interval $t_{\text{CT}}$, set equal to 2 or 9 seconds); and a fixed probe flash presented at 200 ms after the test flash. Here the presumed rod-isolated probe response obtained after computational subtraction of the response to a photopically matched probe flash was similarly analyzed for amplitude. $A(t’)$, the derived rod response to the test flash at time $t’$ after test flash presentation, was determined from the relation

$$A(t’) = A_{\text{rod}}(t’) - A_0(t’) \quad (1c)$$

$$t’ = 200 \text{ ms} + t_{\text{det}} \quad (1d)$$

where $A_{\text{rod}}(t’)$ is the amplitude of the rod-isolated probe response determined in the multi-flash trial, and $A_0(t’)$ is the rod-isolated probe amplitude determined on setting the test flash strength to zero in an otherwise identical trial. $A_{\text{rod}}$ thus represents the probe-alone response prevailing at time $t’$ after the conditioning flash.29

**Dark Adaptometry**

Measurements of visual thresholds were performed with use of a Goldmann-Weekers dark adaptometer and used procedures similar to those previously described.15,30 Briefly, the test stimulus was a flickering light (0.5 Hz) of short wavelength ($\lambda_{\text{max}}$ near 460 nm), and thresholds for detection were measured using the method of limits (both ascending and descending stimulus strengths). The test stimulus subtended a 2° area, and its location in the inferior visual field ranged from 15° to 45°. For each patient, the test stimulus was positioned to illuminate an area outside a central scotoma determined with kinetic Goldmann perimeter and a II2e target. After pupil dilation and 45 minutes of dark adaptation, thresholds were repeatedly measured until a stable value was obtained. The test eye was then exposed to white light (3.1 log cd m$^{-2}$; 5 minutes in duration) that bleached approximately 67% of the rhodopsin, and the subsequent course of dark adaptation was determined.

**RESULTS**

**Genotype Data and a-Wave Characteristics**

Table 1 summarizes genotyping results obtained from the Stargardt patients. For patients 106 to 109, two ABCA4 sequence variations felt to be disease-causing were detected and presumed collectively to be on different alleles. Only a single variation was detected for patient 110. All of the allelic variations in the ABCA4 gene found in these patients are significantly more prevalent in patients with Stargardt disease than in the normal population.24 The ala1038val and gly1961glu alleles are relatively common in Stargardt disease and may have altered frequencies in different populations as a result of founder effects.31
Waveform L2: response to a photopically matched long-wavelength subject 104 with use of the C 670 conditioning forth, the term PAD will be taken to represent the dark-adapted of the response to the probe. 3B, which was taken to represent the rod-mediated component probe-alone response; and waveform LD is the response to a times after the C670 raw probe responses obtained from subject 102 at varying waves of (F) shown in peak-normalized form. The illustrated responses are those obtained with fprobe ≥ 4 seconds. (D-F) Data obtained from normal subject 104 with use of the C670 conditioning flash. Format of the data is similar to that in (A-C).

Experiment 1

Experiments classified as type 1 were conducted to investigate the kinetics of recovery for the rod response to the conditioning flash (Fig. 1). Figures 3A–F show probe responses recorded in representative experiments of this type conducted on two normal subjects (102 and 104) with use of the 670 sc cd s m⁻² conditioning flash (henceforth termed C670). Figure 3A shows raw probe responses obtained from subject 102 at varying times after the C670 flash. Here, each numerical label identifies the interval, in seconds, between the conditioning flash and the labeled probe response; waveform PA, is the dark-adapted probe-alone response; and waveform LD is the response to a photopically matched long-wavelength flash presented under dark-adapted conditions. Computational subtraction of waveform LD from waveform PA yielded response PA, in Figure 3B, which was taken to represent the rod-mediated component of the response to the probe flash (see, e.g., Ref. 22). (Henceforth, the term PA, will be taken to represent the dark-adapted probe-alone response after subtraction of the cone contribution as determined under dark-adapted conditions.) Waveform L2 in Figure 3A is the response to the photopically matched long-wavelength probe presented 2 seconds after the conditioning flash in this experiment. This waveform was computationally subtracted from the probe waveforms obtained at early times after the conditioning flash (for subject 102, waveforms 1 and 2) to yield the corresponding rod-mediated probe responses. Here the L2 response was used as the reference since it was obtained under conditions most similar to those of the early time short-wavelength probe responses. For waveforms 3 to 9 in Figure 3A, cone correction of the raw probe response involved computational subtraction of waveform L2 obtained under dark-adapted conditions. This analysis assumes that the cone response was fully recovered by 2 seconds after the conditioning flash. As illustrated by the peak-normalized waveforms in Figure 3C, the rod-isolated probe responses obtained at middle and late stages of recovery from the conditioning flash exhibited rising-phase kinetics similar to those of the dark-adapted probe-alone response PA, 22,28 Figures 3D, 3E, and 3F show the results of a similar analysis of probe response data obtained from a second normal subject (104). The rod-isolated probe responses of B and E show a growth with time, reflecting recovery of the derived rod response to the conditioning flash. Furthermore, the results shown in Figures 3C and 3F indicate a similarity of response kinetics within each family of responses. Figures 4A–F illustrate response recovery in type 1 experiments conducted with Stargardt patients 109 and 107. As in Figure 3, the data show raw probe responses obtained after the C670 flash (Figs. 4A, 4D), the rod-isolated responses obtained after cone correction (Figs. 4B, 4E), and the peak-normalized rod-isolated responses (Figs. 4C, 4F).
Reciprocal data obtained from each subject were normalized to the amplitude of the dark-adapted probe alone response obtained from that subject. Figure 5A shows the time course of \( A(t)/A_{\text{mod}} \), the normalized derived rod response, as a function of the conditioning-probe interval \( t_{\text{probe}} \). Open symbols indicate results obtained with use of the 670 sc cd s \(^{-2}\) conditioning flash. These data were obtained from the cone-subtracted responses obtained from normal subjects 102 and 104 (Fig. 5), and from similarly analyzed probe responses obtained in identical experiments on two other normal subjects. The data show that a condition of near-saturation of the rod response persisted for approximately 2 to 3 seconds after the flash. These data were obtained from the cone-subtracted responses obtained from normal subjects 102 and 104 (Fig. 5), and from similarly analyzed probe responses obtained in identical experiments on two other normal subjects. The data show that a condition of near-saturation of the rod response persisted for approximately 2 to 3 seconds after the conditioning flash, and half-recovery occurred at approximately 5 to 6 seconds after the conditioning flash. Furthermore, there was an apparent slowing of response recovery during the latter phase of the investigated period. The time course of the recovery determined with the C670 flash can be described by a nested exponential function \( E(t) \), in which \( E(t) \), the excitation generated by the conditioning flash, is presumed to decay through a rapid initial reaction and a subsequent slower reaction (cf., e.g., equations 5 and 6 of Ref. 32).

\[
A(t)/A_{\text{mod}} = 1 - \exp[-\gamma_1 E(t)] \tag{2a}
\]

\[
E(t) = \exp(-t/\tau_1) + \gamma_2[\exp(-t/\tau_2) - \exp(-t/\tau_1)] \tag{2b}
\]

\[
A(t)/A_{\text{mod}} = 1 - \exp[-\gamma_3[\exp(-t/\tau_1) + \gamma_4\exp(-t/\tau_2)]] \tag{2c}
\]

where \( \gamma_1 - \gamma_4 \) are dimensionless constants and \( \tau_1 \) and \( \tau_2 \) are exponential lifetimes. The solid curve in Figure 5A, which plots equation 2c with \( \gamma_3 = 14.72, \gamma_4 = 0.040, \tau_1 = 1.29 \) seconds, and \( \tau_2 = 22.86 \) seconds, closely approximates the data. Also shown in Figure 5A are results obtained from two normal subjects with the 67 sc cd s \(^{-2}\) conditioning flash, henceforth termed the C670 flash (filled symbols). Here, there was no prolonged period of response saturation, and half-recovery occurred at approximately 2 seconds. The dashed curve associated with these data plots equation 2c with \( \gamma_3 = 5.86, \tau_1 = 0.79 \) seconds, and \( \gamma_4 = 0.015, \tau_2 = 1.14 \) seconds, and \( \tau_2 = 82.22 \) seconds. Thus, the time course of recovery from the 670 sc cd s \(^{-2}\) conditioning flash determined for the Stargardt patients was qualitatively similar to that determined for the normal subjects, but characterized by an exponential time constant \( \tau_j \) that exceeded, by more than threefold, that determined for the normal subjects (see the Discussion section). Filled circles in Figure 5B represent results obtained from Stargardt patients 109 and 107 (Fig. 4) and from two other patients. The equation 2c function was fitted to the three sets of data that were relatively closely grouped (circles, squares, and inverted triangles; the omitted data (triangles) were those obtained from patient 108), yielding the illustrated solid curve, with \( \gamma_3 = 22.24, \gamma_4 = 0.015, \tau_1 = 1.14 \) seconds, and \( \tau_2 = 82.22 \) seconds. Thus, the time course of recovery from the 670 sc cd s \(^{-2}\) conditioning flash determined for the Stargardt patients was qualitatively similar to that determined for the normal subjects, but characterized by an exponential time constant \( \tau_j \) that exceeded, by more than threefold, that determined for the normal subjects (see the Discussion section). Filled circles in Figure 5B represent results obtained from a single patient with Stargardt disease with use of the C670 flash. The dashed curve accompanying these results plots equation 2c with \( \gamma_3 = 13.71, \tau_1 = 0.35 \) seconds, and \( \gamma_4 = 0.015, \tau_2 = 1.14 \) seconds, and \( \tau_2 = 82.22 \) seconds.
obtained in type 2 experiments conducted with one normal subject and two Stargardt patients. Shown in the left, middle, and right panels in Figure 6 are, respectively, results obtained under dark-adapted conditions (Figs. 6A, 6D, 6G), results obtained with conditioning flash \( C_{67} \) and a conditioning-test interval \( t_{CT} \) of 2 seconds (Figs. 6B, 6E, 6H), and results obtained with conditioning flash \( C_{670} \) and \( t_{CT} \) of 9 seconds (Figs. 6C, 6F, 6I). In all cases, the test flash of variable intensity and the fixed probe flash were separated by a fixed interval of 200 ms (Fig. 1), a period determined in photocurrent and paired-flash ERG studies to correspond with a time shortly after the peak of the weak-flash response.22,23,35 Shown within each family of responses is the dark-adapted probe-alone response (PA), and response PA, which was obtained in the absence of test flash presentation. Response PA thus represents the probe-alone response that prevailed under the investigated experimental conditions.

Figures 7 and 8 show normalized amplitude-intensity functions determined from probe response data of the types shown in Figure 6. For a given subject and under a given experimental condition, determination of the function involved normalization of the rod-isolated probe amplitudes to the rod-isolated amplitude of the prevailing probe-alone response PA. For combined illustration of the data obtained from different subjects, test flash strengths in these experiments were converted to units of sc td s. Each resultant function describes, as a function of log test flash strength, the amplitude \( A(t) \) of the derived rod response normalized to the prevailing maximum amplitude \( A_{max} \) (see the Methods section). Figures 7A–C and 8A–C show results obtained from the normal subjects and Stargardt patients under dark-adapted conditions (A), with conditioning flash \( C_{67} \) and \( t_{CT} = 2 \) seconds (B), and with conditioning flash \( C_{670} \) and \( t_{CT} = 9 \) seconds (C). Curves fitted to the data of each panel plot the Naka-Rushton relation

\[
A(t')/A_{max} = I_{test}(I_{test,0.5} + I_{test})^{-1}
\]  

where \( I_{test} \) is the test flash strength, \( A_{max} \) is either \( A_{max,D} \) or \( A_{max,L} \), and \( I_{test,0.5} \) is the test flash strength at half-saturation of the normalized function. The determined \( I_{test,0.5} \) thus represents a measure of rod sensitivity to weak (i.e., sub-saturating) test flashes. In both Figures 7 and 8, the fitted curves in A–C are reproduced in D for comparison with one another. Analysis of these functions yielded 1.48 and 1.46 log sc td s for the group average log \( I_{test,0.5} \) determined under dark-adapted conditions from, respectively, normal subjects and Stargardt patients.

Corresponding analysis of the results obtained with the \( C_{670} \) conditioning flash and \( t_{CT} = 2 \) seconds yielded 2.00 and 1.96, respectively, for the group average log \( I_{test,0.5} \) among normal subjects and Stargardt patients. Those obtained with the \( C_{670} \) conditioning flash and \( t_{CT} = 9 \) seconds yielded group-average log \( I_{test,0.5} \) values of 1.92 and 2.14, respectively, for normal subjects and Stargardt patients. Thus, for both groups of subjects, the decreases in rod sensitivity determined with the \( C_{670} \) conditioning flash and \( t_{CT} = 9 \) seconds were approximately 0.5 log unit on average.

Table 2 shows log \( I_{test,0.5} \) determined by the separate fitting of equation 3 to the data obtained from a given subject under the three investigated conditions. For each of these conditions there was no significant difference between the values determined for normal subjects and Stargardt patients (\( P = 0.96, 0.92, \) and 0.50, respectively, for the top, middle, and bottom rows of Table 2). For each subject for whom log \( I_{test,0.5} \) was determined under both dark-adapted conditions and at 9 seconds after the \( C_{670} \) conditioning flash (two normal subjects and four Stargardt patients), the values of log \( I_{test,0.5} \) obtained under the two conditions were also compared through a paired \( t \)-test. This analysis indicated a significant difference between the values of log \( I_{test,0.5} \) determined for the Stargardt patients (\( P = 0.011 \)), but not between those determined for the normal subjects (\( P = 0.128 \)). A similar subject-by-subject, paired \( t \)-test analysis of data obtained under dark-adapted conditions and at 2 seconds after the \( C_{67} \) conditioning flash showed significant differences for the data obtained from two normal subjects (\( P = 0.005 \)) and four Stargardt patients (\( P = 0.007 \)).

**Psychophysically Measured Dark Adaptation**

Open circles, filled triangles, filled circles, and open triangles in Figure 9 show the dark adaptometry data obtained, respectively, from Stargardt patients 106, 108, 109, and 110 after a 67% bleach.15,30 The additional sets of data (solid lines) are results obtained from four normal subjects, one of whom was subject 105 tested in the ERG experiments. For subjects 105, 108, 109, and 110, the dark adaptometry data were obtained from the same eye as that tested in the ERG experiments. Log threshold data obtained from a given subject are referred to the prebleaching (i.e., fully dark-adapted)
threshold determined for that subject (Fig. 9, horizontal dashed line). By comparison with results obtained from the normal subjects, the data obtained from Stargardt patients 106, 109, and 110 showed a modest delay in the final phase of recovery. Those obtained from the fourth patient (subject 108), the individual for whom dark-adapted maximal a-wave amplitude and sensitivity of the amplitude-intensity function were relatively low (Table 1, Fig. 8) and for whom rod recovery kinetics were relatively slow (Fig. 5B and accompanying text), showed a more substantially prolonged course of dark adaptation. Overall, the results obtained from Stargardt patients 106, 109, and 110 indicate a modest but discernible prolongation of desensitization during the final portion of recovery to preillumination baseline.

**DISCUSSION**

The results of this study describe properties of rod dark adaptation in normal subjects and in ABCA4-associated Stargardt patients, after a weak-bleaching but rod-saturating conditioning flash. Rod recovery in human subjects after exposure to bright conditioning light has been previously investigated through measurement of the ERG a-wave. To our knowledge, however, the present study is the first to employ the paired-flash method to determine the instantaneous rod amplitude-intensity function (and thus, the sensitivity parameter $I_{test,0.5}$) during dark adaptation in human subjects (type 2 experiments). In the present experiments, we focused on sensitivity determinations at fixed intermediate times associated with recovery of a substantial portion of the maximum, dark-adapted range of the photoresponse (i.e., with recovery of the response to levels well below saturation). A main conclusion from these sensitivity determinations is that at 2 seconds after the C67 conditioning flash and at 9 seconds after the C670 flash—that is, at times when response recovery is near complete (cf. Fig. 5)—there is a remaining significant desensitization of the rod response function in both normal subjects and Stargardt patients. Furthermore, as determined both with $t_{CT} = 2$ seconds after the C67 flash and $t_{CT} = 9$ seconds after the C670 flash, desensitizations of the normalized amplitude-intensity functions exhibited by the investigated Stargardt patients are similar to those exhibited by the normal subjects (Figs. 7, 8).

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**FIGURE 6.** Rod-isolated probe responses obtained in paired-flash trials during recovery from a conditioning flash (experiment 2). (A–C) Data from normal subject 102 obtained with test flashes over the range of 4.36 to 437 sc td s. (D–F) Data from Stargardt patient 109 obtained with test flashes over the range of 3.46 to 347 sc td s. (G–I) Data from Stargardt patient 110 obtained with test flashes over the range of 3.46 to 347 sc td s. Data in (A), (D), and (G) were obtained under dark-adapted conditions; data in (B), (E), and (H) were obtained with conditioning flash C67 and $t_{CT} = 2$ seconds; data in (C), (F), and (I) were obtained with conditioning flash C670 and $t_{CT} = 9$ seconds. PA0 is the dark-adapted probe-alone response; PA is the prevailing probe-alone response. Numerical labels shown in (A), (D), and (G) identify test flash strengths associated with the indicated probe responses. Scale bars shown in (A), (D), and (G) refer, respectively, to data obtained from subjects 102, 109, and 110.
These results leave open the possibility that the period required for full recovery of rod sensitivity exceeds that required for the near-completion of excitation decay. However, properties of the data obtained from the group of trials conducted within a given recording session (i.e., groups of amplitude-intensity data that are generally well described by the Naka-Rushton equation) argue against the possibility that any such period of lingering desensitization exceeded the approximately 1-minute period that separated consecutive trials. Furthermore, despite the lower average a-wave maximum amplitude (Table 1) and the apparently slower recovery from the C670 conditioning flash (described later) determined for the Stargardt patients, occurrence of an ABCA4 mutation in these patients does not substantially affect dark-adapted rod sensitivity (Figs. 7, 8, and accompanying text; Table 2).

In both normal subjects and Stargardt patients, recovery from the C670 conditioning flash was characterized by a biphasic process generally similar to those observed in previous studies of electroretinographically determined rod recovery (e.g., Refs. 28, 36, 37). Specifically, analysis of the data of Figure 5 through the present equation 2 indicates that rod recovery from the C670 flash both in normal subjects and in the investigated Stargardt patients is consistent with the partial deactivation of a photoactivated intermediate on a time scale of approximately 1 second (1.29 seconds for normal subjects, 1.14 seconds for Stargardt patients; see text accompanying Fig. 5), and further deactivation through a process occurring on a considerably longer time scale (described later). The equation used here to model recovery is identical with that used to characterize the falling phase of the rod response after weak (nonbleaching) stimuli28 and significant rhodopsin bleaching,29 except for the substitution of the function E(t) (equation 2b) for the single term used previously. As E(t) describes the decay of an activated species through two sequential first-order reactions, the fit provided by equation 2 is consistent with the occurrence of two sequential first-order deactivation reactions that rate-limit recovery of the response (cf. Refs. 36, 37).

The equation 2 fit to the data obtained with the C670 conditioning flash yielded markedly different values for the slower of the two time constants (τ2 = 22.86 seconds for normal subjects; 82.22 seconds for Stargardt patients). It is of interest to consider this difference in light of the finding that dark-adapted values of weak-flash sensitivity for the two groups of subjects are similar (e.g., Table 2). By facilitating the translocation of all-trans retinal (as N-retinylidene-phosphatidylethanolamine38) from the disc lumen to the cytosol of the rod outer segment, the ABCA4 gene product is thought to facilitate the removal of all-trans retinal from opsin and thereby promote the termination of excitation in the phototransduction cascade (see the Introduction). Thus, a possible basis for the relatively slow late phase of electroretinographically determined response recovery in the investigated Stargardt patients after the C670 conditioning flash (i.e., the relatively high value of the fitted τ2) is a sluggish removal of all-trans retinal from the disc lumen. On this interpretation, the general similarity of the dark adaptometry results obtained from the normal subjects and Stargardt patients after extensive bleaching (i.e., the occurrence in three of the Stargardt patients of a modest delay,
relative to normal subjects, only in the final phase of dark adaptation; Fig. 9) can be explained on the hypothesis that with large bleaches, the amount of all-trans retinal photoproduct present in the disc lumen is sufficient, over all but the final stage of recovery, to saturate (i.e., overwhelm) the all-trans retinal removal process in the rods of normal subjects as well as Stargardt patients, and thus to produce a relative similarity in the time courses of dark adaptation.

**TABLE 2.** Determinations of Logarithmic Weak-flash Sensitivity (log $I_{test,0.5}$)

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Normal Subjects</th>
<th>Stargardt Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark adapted</td>
<td>1.48 ± 0.22 (4)</td>
<td>1.47 ± 0.32 (5)</td>
</tr>
<tr>
<td>$C_{67}$, $t_{CT} = 2$ s</td>
<td>1.99 ± 0.17 (2)</td>
<td>1.96 ± 0.32 (4)</td>
</tr>
<tr>
<td>$C_{670}$, $t_{CT} = 9$ s</td>
<td>1.95 ± 0.16 (4)</td>
<td>2.14 ± 0.30 (4)</td>
</tr>
</tbody>
</table>

Values of log $I_{test,0.5}$ (mean ± SD) determined by fitting equation 3 to amplitude-intensity data obtained from individual subjects. Entries in parentheses indicate the number of subjects. Averages determined in this subject-by-subject analysis differ slightly from the group average data noted in the text accompanying Figures 7 and 8. The difference is due to the nonlinear nature of the fitting procedure used to determine log $I_{test,0.5}$ for a given subject.

**FIGURE 8.** Amplitude-intensity functions obtained from Stargardt patients. The format is as in Figure 7. Identical symbols indicate data obtained from the same subject. Data obtained from five patients in (A), four in (B), and four in (C). Curves plot equation 3 fitted to the data. Inverted triangles: data obtained from patient 108. In (A) the circled inverted triangle was omitted in the curve fitting. (D) Fitted curves reproduced from (A–C).

**FIGURE 9.** Psychophysically determined time course of dark adaptation in four normal subjects and four Stargardt patients after illumination estimated in normal subjects to produce approximately 67% bleaching. The four segmented lines indicate results from four visually normal subjects, one of whom was subject 105. Log threshold data obtained from a given subject are referred to the prebleaching (i.e., fully dark-adapted) threshold determined for that subject (horizontal dashed line).
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References


