Photonic modulation of a highly sensitive, near-infrared light-scattering signal recorded from intact retinal photoreceptors

(phototransduction/vision)

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ABSTRACT On stimulation by green flashes, the isolated, aspartate-treated bovine retina exhibits transient changes in the scattering of near-infrared (880 nm) light. A single component, termed the "ATR" (a flash-induced scattering signal, where ATR designates amplified transient-retina), dominates the amplitude and rising-phase kinetics of the initial peak of the light-scattering response. Superfusion with physiological solution containing low Na+ concentration reversibly abolishes the photoreceptor electroretinographic response but preserves the ATR signal, indicating a receptoral origin for the ATR. The increase of ATR amplitude (A/A_max) with flash intensity (R*/R, where R indicates rhodopsin) is described by A/A_max = (1 - e^{-AR*/R}) with R* = k^{-1} occurring on generation of approximately two photoactivated rhodopsins (R*) per disc surface in the rod outer segment. Weak background light and bright flashes reversibly depress the ATR. Kinetic and sensitivity data suggest a basis of the effects of varying illumination. A delay, apparently governed by the lifetime of a light-activated substance regulating ATR generation, precedes ATR recovery after a bright flash. The flash dependence of the delay period indicates an upper limit of 3 s for the lifetime of R* in the ATR-generating process. The unit event appears to be an R*-catalyzed and disc-localized reaction of phototransduction.

The high electrophysiological sensitivity of retinal rod photoreceptors reflects amplification of the photic signal at multiple steps of the transduction process. The first amplifying stage operates on the disc membranes of the receptor outer segment and involves the catalytic activation of guanine nucleotide-binding protein (G protein) by photoactivated visual pigment (photoactivated rhodopsin, R*). Activated G protein (G*) promotes activation of cGMP phosphodiesterase (PDE) on the disc membranes (forming PDE*). Through these reactions, a single R* induces transient activation of many G proteins and PDEs within a local region of spatial extent approximately one disc surface (reviewed in ref. 1).

In rod outer segment (ROS) preparations and intact isolated retinas, test flashes produce minute structural changes that can be monitored by light-scattering (LS) at near-infrared wavelengths (2-7). Among the several types of LS signal described previously is the amplified, transient ("AT") response recorded from permeabilized bovine ROS (8, 9). The AT signal appears to monitor the activation of PDE—i.e., G* + PDE- → G*1 + PDE*, where I is the inhibitory subunit of PDE (1, 10).

Electrophysiological data indicate that conditioning light (sustained background or bright flash) influences operation of one or more intermediate reactions of phototransduction (11–14). To elucidate the nature of these changes, it would be desirable to measure, within intact receptors, the effects of illumination on distal, amplifying reactions. Here we report that electrophysiologically active rod photoreceptors of the bovine retina exhibit a flash-induced scattering signal ("ATR" signal; abbreviation for "AT-retina"). The stability of which allows quantitative study over periods of several hours. With respect to photic sensitivity, and catalytic role of R* in stochastic unit activations, the process investigated here exhibits a remarkable similarity to PDE activation, studied in vitro by Liebman and Pugh (15, 16). Some of our results were reported at the 1987 meeting of the Biophysical Society (17).

METHODS

Bovine eyes obtained from a local abattoir were immediately protected from light. Dissection and preparation of the isolated retina typically were initiated within 1 hr of the animal’s death and carried out under dim red light. Circular sections of retina (diameter, 7 mm) were isolated from the back of the eye, positioned in a transparent cylindrical chamber (volume, 1 mL), and continuously superfused with oxygenated physiological medium (flow rate, 1 mL/min; ambient temperature, 20–22°C). The standard medium, modified from that described (18), contained NaCl, 134; KCl, 2.0; CaCl2, 0.1; MgCl2, 0.1; NaH2PO4, 1.5; Na2HPO4, 4.2; glucose, 5.0; and l-aspartate, 2.0; adjusted to pH 7.2. Low-Na+ medium was prepared by substituting 134 mM choline chloride for the NaCl (19).

Measurements of LS employed a continuous beam of 880-nm light from a light-emitting diode (Hitachi HLP 60R). The incident infrared (IR) beam was normal to the plane of the retina and entered at the vitreal surface. Light scattered within an annular region (interval of angles θ referred to the forward optical axis) was collected by a Fresnel lens system and focused onto a solid-state photodetector (Centronic OBD 100 S-IT) (8, 20). Markers accompanying LS waveforms identify units of (ΔI)/I, the relative, flash-induced change in the intensity of scattered IR light. The standard configuration (scattering angle, 6° < θ < 10°) was altered for analysis of the angular dependence of LS signals.

Stimulating flashes of green light (20 μs in duration; E.G. & G. FX-133 flashlight) and continuous, green background light were delivered to the retina by a fiber optic guide.

Abbreviations: G protein, guanine nucleotide-binding protein; PDE, phosphodiesterase; ROS, rod outer segment; AT, amplified transient; ATR, AT-retina; LS, light-scattering; IR, infrared; ERG, electroretinographic; L.A., light-adapted; D.A., dark-adapted; R, rhodopsin.

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positioned at an inclination of 10° relative to the incident IR beam. Intensities of the flash and background beams (\(\lambda_{\text{max}} = 520 \text{ nm}\)) were independently attenuated by neutral density filters. Efficiencies of rhodopsin photoactivation (bleaching) were determined by spectrophotometry of intact retinas. Flash intensity is expressed as a fractional bleach, \(R^*/R\); the intensity of background light, \(I_{\text{bg}}\), is expressed as \((R^*/R) \text{ s}^{-1}\). The term, “dark-adapted” (DA), will refer to the state of the retina before, or long after, exposure to conditioning light (steady background or bright flash). “Light-adapted” (LA) will refer to the state prevailing during background illumination. “Sensitivity” of the ATR scattering signal will be represented by \(k\), a parameter characterizing the amplitude response function (see Eq. 1, below). The value of \(k\) is defined as the inverse of the flash intensity yielding 63% \((1 - e^{-k})\) of the prevailing maximal amplitude of the ATR.

 Routinely, two or three flash responses were averaged for analysis of LS signals (Nicolet 2900-III A recorder, and off-line averaging by computer). The preflash segment of the record (typically, 20% of the sweep) was used as the baseline. Baseline drift was set to zero by a computer program that calculated the slope of this preflass segment. The interval between flashes was sufficient to avoid conditioning by the test flash; quoted times of measurement identify periods of data collection (typically, several minutes). Experiments involving paired flashes (conditioning and probing stimuli) employed electronic timing of the interflash interval, to afford averaging of data from two or three runs. The interflash interval exceeded the period (<0.4 s) required for recharging of the flashgun.

 Electroretinographic (ERG) PIII responses, isolated by the presence of L-aspartate in the superfusion medium (21), were recorded, simultaneously with LS signals, by transectinally positioned Ag/AgCl electrodes. Responses were amplified (bandpass, DC-1 kHz) and averaged in conventional manner. Recordings were analyzed for amplitude at a poststimulus time of 1.3 s; examination of the waveforms indicated domination of the measured response by the rod component at this time. Half-saturation of the rod response in DA retinas occurred at \(R^*/R \approx 2 \times 10^{-7}\).

 The number of rhodopsin molecules on a single disc surface was assumed to be \(3 \times 10^{11}\). This estimate is based on a disc diameter of about 1.2 \(\mu\text{m}\) (electron microscopic measurements; K.P.H., unpublished observations) and a rhodopsin density of about 25,000 per \(\mu\text{m}^2\) (22).

**RESULTS**

**Cellular Origin.** Flash stimulation of the aspartate-treated retina elicited a LS response and an ERG response (Fig. 1A, top row). The LS signal contained an initial deflection representing an increase in scattering (positive-going wave). Later sections will analyze the sensitivity and rising-phase kinetics of this initial peak, which were generally similar among preparations. Occurrence of the initial peak in aspartate-treated retinas renders likely an origin of the response in the photoreceptors or Muller cells (21). Evidence that this signal monitors a photoreceptor process came from experiments employing low-Na\(^+\) medium to eliminate the light-sensitive current of the receptors (19). When substituted for the standard superfusion medium, the low-Na\(^+\) medium abolished the ERG response but preserved early components of the LS signal, including the initial peak (Fig. 1A). A slowly developing, negative-going phase of the LS response was reversibly abolished by low-Na\(^+\) medium (data not illustrated).

The photoreceptor ERG response (early PIII) of mammalian retinas consists of a dominant, rod component and a minor, rapidly decaying component (“nose”) due to cone activity (23). In a preparation exhibiting a prominent cone ERG component, we asked whether background-induced reduction of the LS signal preserves the ERG contribution from a particular receptor type (Fig. 1B). Under conditions that suppressed the LS response, ERG data indicated persistence of a significant fraction of the DA cone response and the absence of a substantial rod response. This finding suggests that the initial phase of the LS signal arises largely or entirely from events within the rods.

**Angular Dependence.** With respect to time course and amplitude, the later course of the LS signal varied among preparations. The two retinas illustrated in Fig. 2 exemplify the range of characteristics observed for the decline from the initial, positive deflection. In the case of retina 1 (representative of the more frequently observed behavior), comparison of peak-normalized records obtained at different scattering angles indicated a similarity of rising-phase kinetics but significant variation in kinetics of the falling phase. This result indicates the contribution of distinct processes (multiple components) to the measured response. Some preparations (retina 2 in Fig. 2) exhibited a relatively gradual decline from the initial peak. Recordings from these preparations appeared kinetically similar over a range of scattering angles. Such uniformity argues for a single process as the underlying basis of the signal. The data of Fig. 2 together indicate that a single process largely governs the amplitude and rising-phase kinetics of the initial LS peak and that,
FIG. 2. Angular dependence of the LS response to a fixed stimulating flash \((R*/R = 1.6 \times 10^{-4})\). Signals were recorded from retinas 1 (left) and 2 (right) within the indicated intervals of scattering angle (θ); overall periods of measurement were 125 min (retina 1) and 91 min (retina 2). Each set of signals is scaled for equal peak amplitude (normalized). Each set of responses includes a second, control measurement at a single angular interval (bottom row: 3–6° and 6–10°, respectively, for retinas 1 and 2); the period between the first and second measurements at this interval was >90 min.

Varially among preparations, at least one other process influences the subsequent time course of the response. The initial LS transient will henceforth be termed the ATR signal.

**Photic Sensitivity.** Amplitude of the ATR was graded with intensity \((R*/R)\) of the stimulating flash (Fig. 3). Relative amplitudes \((A/A_{\text{max}})\) measured under DA conditions were well described by the relationship,

\[
A/A_{\text{max}} = 1 - e^{-kR*/R},
\]

[1]

where the sensitivity parameter, \(k\), represents the inverse of the fractional bleach at \(A/A_{\text{max}} = 1 - e^{-1} = 0.63\) (Fig. 4, filled circles). Rate of development of the ATR (slope of the rising phase) increased with \(R*/R\) roughly in linear fashion throughout the range examined (Fig. 4, open circles). Determinations of \(k\) for seven DA retinas yielded \(k_{DA} = 18,200 \pm 8,300\) (mean ± SD), equal to about one-half of the rhodopsin population \((3 \times 10^6)\) of a single disc surface in the ROS. Thus, at \(R*/R = k^{-1}\), the stimulating flash generates approximately two R's per disc surface. This value of \(R*/R\) exceeds, by \(>10^2\)-fold, the flash intensity at half-saturation of the ERG response.

**Background Illumination.** Weak background light depressed the ATR to a superimposed test flash (Figs. 3 and 4). Under all but extreme conditions of background intensity or duration, nearly complete recovery of the ATR occurred on extinction of the background (see Fig. 1B). As in the DA condition, relative ATR amplitude \((A/A_{\text{max, DA}})\) in the presence of the standard background \((I_b = 6.2 \times 10^{-6} R^*/R s^{-1})\) exhibited a dependence on \(R*/R\) given by Eq. 1 (Fig. 4). The background preserved the general waveform (Fig. 3) and rising-phase kinetics (Fig. 4) of the DA response and had no substantial effect on the value of \(k\) \((k_{DA}/k_{LA} = 1.1 \pm 0.4; n = 4)\). Cumulative rhodopsin bleaching by the background light typically was <4%, ruling out depletion of rhodopsin as a cause of the reduction in amplitude.

The conditioning effect of background-generated R's, reflected in the depression of maximal ATR amplitude, may be described by a function \(f(t)\) with “integration time” \(\tau_i \approx \int_0^t f(t)dt\). With Eq. 1, we may write \((A_{\text{max, DA}} - A_{\text{max, LA}})/A_{\text{max, DA}} = 1 - e^{-k\tau_i}\). Thus,

\[
A_{\text{max, LA}}/A_{\text{max, DA}} = e^{-k\tau_i}. \tag{2}
\]

Data obtained in four experiments yielded \(\tau_i = 8.2 \pm 2.4\ s\).

**Conditioning Flashes.** Bright flashes [intensity \((R^*/R)_f\)] transiently depressed the ATR elicited by a second, probing flash of the same intensity (Fig. 5 A and B). By comparison with the response to the first flash, those recorded immediately after the stronger conditioning flashes exhibited altered overall waveform and little or no ATR component (Fig. 5B). The period of virtually complete suppression of the ATR (delay period) was followed by a period in which ATR amplitude and overall waveform of the response gradually approached those of the nominal signal. Duration \((T_d)\) of the delay period increased logarithmically with \((R^*/R)_f\) (Fig. 5A, Inset).
The late-stage recovery of ATR amplitude proceeded in approximately exponential fashion, with characteristic time $\tau_1$ (Fig. 5A). Among preparations, values of $\tau_1$ for the range, $(R^*/R)_c = 0.7 \times 10^{-4}$-2.7 $\times 10^{-4}$, did not differ significantly from one another; these values, $\sim 10$ s, were roughly similar to values of $\tau_1$ (Fig. 5C). In one retina displaying kinetically similar LS responses over a range of scattering angles (Fig. 5D: same preparation as retina 2 of Fig. 2), recovery of the ATR response proceeded in parallel with the decline from peak of the response to the conditioning flash. Measurements of $\tau_1$ and $\tau_2$ for this retina yielded 7.2 and 7.8 s, respectively.

**DISCUSSION**

Our results show that the ATR signal monitors a light-activated, transient process within functioning rod photoreceptors. Generation of the ATR does not require development of the rod ERG response. Thus, the process responsible for the ATR does not depend on the changes in plasma membrane current ordinarily induced by light.

Properties of the ATR suggest a basis in unit activation events that are initiated by single, catalytically functioning R*s and that extend over approximately one-half of a disc surface. We base this conclusion on the roughly linear dependence of rate of rise on $R^*$ and on the observed value of the sensitivity parameter $k$ ($k(R^*/R) = 1$ on generation of approximately two $R^*$ per disc surface). The exponential nature of the amplitude response function furthermore suggests Poisson-distributed and therefore, independent, unit events—that is, the measured ATR ($A/A_{\text{max,DA}}$) represents the superposition of signals generated by $N^*$ unit activations:

$$A/A_{\text{max,DA}} = 1 - e^{-kR^*/R} = N^*/N_{\text{tot}},$$

where $N_{\text{tot}}$ is the total number of units, and $k$ is the average number of rhodopsins serving as potential activators of a given unit (see Eq. 1).
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Interpreted on this view, the results obtained with conditioning light imply a striking property of the unit event—namely, an essential constancy of size and time course under different conditions of illumination. For example, such an invariance predicts the sole effect of background light to be preactivation of units (sustained average activation = "preflash N*") and consequent reduction in the number of units available for activation by a superimposed flash. Consistent with the observed scaling of LA vs. DA amplitude functions, superposition of the effects of a background and flash yields (through Eqs. 2 and 3)

\[
A/A_{\text{max,DA}} = (1/N_m)(\text{"postflash N*"} - \text{"preflash N*"}) = [1 - e^{-\theta(R/R_\theta + t_{\theta})}] - [1 - e^{-k_{\theta}t_{\theta}}] = (A_{\text{max,LA}}/A_{\text{max,DA}})(1 - e^{-kR/R_\theta}). \tag{4}
\]

This notion furthermore accounts for the findings that background light preserves the rising slope and general waveform of the DA response (Figs. 3 and 4) and that recovery time (\(\tau_r\)) is relatively constant over a substantial range of conditioning flash intensities (Fig. 5C).

A key relationship predicted by invariance of the unit event is an equivalence in the conditioning effects of R* generated by a steady background vs. a bright flash—that is, effective lifetime of the light-activated reaction (\(\text{S} \to \text{S}^*\)) directly monitored by the ATR; and that levels of \(\text{X}^*\) exceeding a constant "threshold," \(\text{X}^*_\text{f}\), suppress the response to a probing flash by maintaining the entire complement of \(\text{S}\) in activated, \(\text{S}^*\) form (i.e., by maintaining \(\text{S} = 0\)). At the initiation of recovery, \(t = t_{\theta}, S > 0\), and \(X_{\theta}/\alpha = \text{(R/R}_\theta)\), \(e^{-\theta R/R_\theta}\). The observed linear relationship between \(T_\theta, [\ln(R/R_\theta)]\) is consistent with such a scheme and yields a value of 2.7 s for \(\tau_{\theta}\). Thus, \(\text{X}^*\) itself, and any light-activated species preceding \(\text{X}^*\) in the ATR-generating process, must have a lifetime \(\leq 2.7\) s. The possibility exists that \(\text{X}^* = R^*\).

Properties of the ATR, and its resemblance to previously described LS signals linked with activation of G protein and cGMP PDE, suggest involvement of at least one of these disc-localized proteins in the reaction generating the ATR. Evidence implicating PDE activation as the basis of the ATR comes from comparing time-to-peak of the ATR for weak flashes (\(=0.5\) s; Fig. 3) with the rate of activation of G. The latter number, estimated for in vitro systems by spectrophotometry of metahordopsin II, by exchange rate of GTP, and by LS, is about \(10^3\) s\(^{-1}\) per \(R^*\) (5, 16, 24). Given the abundance of G and PDE on the disc surface (about 3000 G proteins and 300 PDEs) (1) and evidence for high affinity of endogenous G* for PDE (10, 25), a period of 0.5 s is more than sufficient to activate the evident unit complement of PDE (that of one-half disc surface) and insufficient for activation of the unit complement of G. A basis in PDE activation would also explain the similarity between the sensitivity parameter for the ATR \((k = 18,200 \pm 8,300)\) and that describing the Poisson-distributed activation of PDE in bovine ROS disc preparations \((=26,300)\) (15, 16). Finally, the present result resembles the AT signal of isolated, permeabilized bovine ROS with respect to photic sensitivity and general waveform (although time scale of the ATR is much shorter) (8, 9).

Experiments with protamine, which activates PDE but not G protein, indicate that the AT signal monitors generation of PDE* and not, for example, either generation of G* or hydrolysis of cGMP (refs. 8, 26, 27; see, however, ref. 28).

The present results establish LS as a quantitative probe of the rod disc amplifier under physiological conditions and suggest a remarkable constancy of operation of an intermediate transduction step over a range of illumination conditions. LS studies of \(\alpha\)-toxin-permeabilized retina preparations (29), which afford adjustment of nucleotides to defined concentrations, may provide information as to in situ biochemical mechanisms regulating this transduction reaction.

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