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Comparative investigation of stimulus-evoked rod outer segment movement and retinal electrophysiological activity

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Abstract

Transient retinal phototropism (TRP) has been observed in rod photoreceptors activated by oblique visible light flashes. Time-lapse confocal microscopy and optical coherence tomography (OCT) revealed rod outer segment (ROS) movements as the physical source of TRP. However, the physiological source of TRP is still not well understood. In this study, concurrent TRP and electroretinogram (ERG) measurements disclosed a remarkably earlier onset time of the ROS movements (10 ms) than that (~38 ms) of the ERG a-wave. Furthermore, low sodium treatment reversibly blocked the photoreceptor ERG a-wave, which is known to reflect hyperpolarization of retinal photoreceptors, but preserved the TRP associated rod OS movements well. Our experimental results and theoretical analysis suggested that the physiological source of TRP might be attributed to early stages of phototransduction, before the hyperpolarization of retinal photoreceptors.

Keywords

Retina; photoreceptor; visual electrophysiology; functional imaging; ophthalmology

1. Introduction

Obliquely stimulated by a visible light flash, rod photoreceptors in both amphibian (frog) and mammalian (mouse) retinas have been observed to present a transient phototropic change, termed a transient retinal phototropism (TRP) [1]. Functional optical coherence tomography (OCT) further revealed the rod outer segment (ROS) as the anatomic source of TRP [2]. Our recent studies with retina slices confirmed this by directly recording a stimulus-evoked movement of the ROS in both frog and mouse retinas [3]. Although TRP shows a significant physical connection with light-evoked ROS movements, its physiological origin, for example, whether TRP arises from the procedures of phototransduction in ROSs or is a physical response related to the activity of downstream neurons (e.g., bipolar cells and horizontal cells), is still unknown. Previous studies reported an intrinsic near-infrared (NIR) scattering signal from isolated ROSs in response to a visible flash stimulation [4, 5].

Subsequent studies on intact retinal photoreceptors demonstrated the scattering signal has a receptor origin [6, 7]. As the ROS movement introduces a structural change in the retina, it is reasonable to hypothesize that the TRP is a critical component of the light-scattering signal [8]. Therefore, TRPs may also originate from the ROS.

Our previous *in vivo* study of frog retinas demonstrated that the onset time of the intrinsic optical signal (IOS) is earlier than that of the electroretinographic (ERG) a-wave [9]. As the ERG a-wave is well known to reflect the hyperpolarization of photoreceptor outer segments (OSs) [10, 11] and the TRP was speculated to be a primary contributor to the IOS [1], we further hypothesized that the origin of TRP correlates to the phototransduction processes before cell hyperpolarization. To verify this hypothesis, a modified Ringer's medium with sodium ions substituted by choline was employed to suppress the light-evoked hyperpolarization of the ROS [7], and consequently, block the signal transmission to downstream neurons. The low-sodium Ringer's medium shut down the influx of sodium ions into the ROS (known as the dark current) and maintained the ROS hyperpolarized as choline cannot pass through cyclic guanosine monophosphate (cGMP) gated ion channels, and eventually resulted in the downstream neurons insensitivity to light stimulation [10-12]. Therefore, TRP should have a receptor origin if it remains during superfusion with a low-sodium medium. Additionally, if TRP is the result of phototransduction events upstream from the closure of cGMP-gated ion channels, the onset of TRP might be expected to present earlier than that of the ERG a-wave, which principally reflects the reduction of the photoreceptor circulating current.

2. Materials and Methods

2.1 Retinal preparation

The experiments followed the protocols approved by the Animal Care Committee at the University of Illinois at Chicago, and conformed to the statement on the use of animals in ophthalmic and vision research, established by the Association for Research in Vision and Ophthalmology. Leopard frogs (*Rana pipiens*) were selected for our experiment as the relatively large size of the frog photoreceptors enables unambiguous observation of individual photoreceptors and convenient quantitative analysis of ROS movements. The frogs used in this experiment were first dark-adapted for at least eight hours and then euthanized by decapitation and double pithing. The eyes were enucleated and hemisected along the equator with fine scissors. A separation of the retina from the rest of the eye was then conducted and the isolated retina was transferred into a recording chamber with OS of the photoreceptors facing upward. All the procedures were performed in a dark room with dim red light illumination and the retina was continuously superfused with oxygenated Ringer's medium at room temperature (18~20 °C). The Ringer's medium contained 110.0 mM NaCl, 2.5 mM KCl, 1.6 mM MgCl₂, 1.0 mM CaCl₂, 22.0 mM NaHCO₃, and 10.0 mM D-glucose and the pH was maintained at 7.3~7.4. The low sodium medium was prepared by replacing the NaCl in the Ringer's medium with 110.0 mM choline chloride [7].

2.2 System setup

As the schematic diagram shows in Figure 1A, the experimental system was composed of two major parts: image acquisition and electrical signal recording. The acquisition of ROS movements was achieved by imaging the retina with a modified NIR light microscope (BX531WI, Olympus, Japan) and a CCD camera (pixel size: $7.4\ \mu\text{m} \times 7.4\ \mu\text{m}$; Pike, Allied Vision, Exton, PA). The acquisition rate of the camera was set to 100 frames per second (fps) with each frame covering a $\sim 74\ \mu\text{m} \times 74\ \mu\text{m}$ area on the retina (Fig. 2A1-2A3). To detect the onset time of ROS movement, a CMOS camera (pixel size: $6.5\ \mu\text{m} \times 6.5\ \mu\text{m}$; Neo 5.5, Andor Technology Ltd, Belfast, Ireland) was then employed to replace the CCD camera and provided an acquisition rate of 500 fps with each frame covering a retinal area of $\sim 70\ \mu\text{m} \times 19.8\ \mu\text{m}$ (Fig. 4A). The recording of ERGs was implemented by using a full-field ERG recording method [13] with a pair of differential electrodes placed on the top and bottom of the retina (Fig. 1A, dashed square), respectively. The acquired ERG signal (sampling rate: 10 kHz) was amplified (gain: 1,000), bandpass filtered (pass-band: 0.1 Hz $\sim$ 10 kHz), digitalized and eventually sent to the computer for display and storage.

The visible light for retinal stimulation was generated by a fiber-coupled light emitting diode (LED; center: 550 nm, bandwidth: $\sim 200\ \text{nm}$) and then was collimated and delivered to the retina for a $\sim 20^\circ$ (angle between the incident light and the longitudinal axis of photoreceptors, Fig. 1B) oblique stimulation. To better identify the stimulus-evoked ROS movement, the isolated retina was partially illuminated by visible light and the stimulus pattern that was projected onto the retina was a rectangular area of dimensions $\sim 20\ \mu\text{m} \times 252\ \mu\text{m}$ that covered about two rod photoreceptors in the lateral direction (Figs. 2A1, 2B1 and 2C1). The stimulus intensity was empirically set to $\sim 1.13 \times 10^8\ \text{photon}/\mu\text{m}^2/\text{s}$ to enable robust observation of ROS movements. The image acquisition, ERG recording and retinal stimulation were synchronized to fulfill a simultaneous recording of both ROS movements and ERGs before, during and after the stimulation.

2.3 Experimental procedure

To conduct a comparative study of retinal physiological responses, the retina was consecutively superfused with Ringer's solution, low-sodium solution and then Ringer's solution again. Each superfusion period was set to 20 minutes, allowing a complete replacement of the previous superfusion medium and sufficient retinal recovery. A 5.0-s imaging period, including a 1.0-s pre-stimulus phase, a 1.0-s stimulus phase and a 3.0-s post-stimulus phase, was performed immediately after each superfusion period. During the imaging period, the superfusion was suspended to provide a static recording environment, and ROS movements and ERGs were recorded simultaneously.

The onset time of ROS movements was investigated by applying a CMOS camera which provided a temporal resolution of 2 ms. To reflect the general response of ROSs to the stimulus, ROS movements and ERG data were obtained from multiple locations of a retina superfused with Ringer's medium and averaged for data analysis.

2.4 Data analysis

The methods employed for the quantification of ROS movements and the segmentation of active areas were reported in our previous publication [2]. For each acquired retinal image sequence, a magnitude map and a direction map of ROS movements were generated using an optical flow algorithm [14]. In this study, the temporal dynamics of the ROS movement magnitude was represented by the time course of the mean ROS movement magnitude in each retinal image. The calculation of the mean ROS movement magnitude was similar to the method previously employed for the computation of IOS, with specific parameters of a 3- σ threshold (mean plus three standard deviations) and a temporal window of eight frames [9, 15].

3. Results

To investigate the physiological origin of TRPs, light-evoked ROS movements and ERGs were compared under conditions with superfusion of control Ringer's medium and low-sodium medium. As shown in Figures. 2A1-2A3, the imaging area was fixed on the same location of the retina during the recordings to exclude possible retinal response diversities due to different retinal locations. Retinal images (Figs. 2A1-2A3), ROS movement magnitudes (Figs. 2B1-2B3) and direction (Figs. 2C1-2C3) maps acquired at 0.4 s after the onset of stimulation were selected to check retinal activities. Significant ROS movements were observed within the stimulated area and adjacent regions (Figs. 2B1-2B3), and the movement directions were centered near 90° (Fig. 2C1-2C3; coordinates of movement angle are shown in the bottom of Fig. 1B), showing that the stimulated ROS swung toward the direction of the stimulus. The time courses of the mean ROS movement and ERG are shown in Figs. 2D1-2D3 and Figs. 2E1-2E3, respectively. As shown in Figure 2E2, the ERG responses were totally abolished in the experiment with low-sodium medium perfusion. However, ROS movements were consistently observed in both control (Figs. 2D1 and 2D3) and low-sodium (Fig. 2D2) medium experiments. In both control and low-sodium experiments, the waveforms of the time courses of mean ROS movement magnitudes exhibited a stable and flat stage before an instant rise and a peak value at about 0.5 s after the onset of the flash stimulation, indicating that the substitution of low-sodium medium did not substantially affect the process of TRP.

The comparative experiments with control Ringer's medium and low-sodium medium were then conducted on different retinas to investigate the possible effects of retinal diversities on ROS movements and ERGs. For each of the control and low-sodium experiments, six data sets of ROS movements and correspondent ERGs were obtained from six different retinas. The mean and standard deviation of the data sets in each individual experiment were then calculated to reflect a general retinal response to the stimulus. As shown in Figure 3, the ROS movement persisted in both control and low-sodium perfusion experiments, but ERG responses vanished in the low-sodium medium group. Additionally, the time courses of both the mean ROS movement and ERG data from different retinas presented general similarity in overall shape and peak attributes.

To further illustrate the timing relationship between the onsets of light-evoked ROS movements and ERG a-waves, retinal images were recorded from a reduced image area,

with improved temporal resolution (2 ms; Fig. 4A). Figure 4B shows representative magnitude maps of the ROS movement acquired in a temporal sequence. To better identify the onset time, signal denoising was performed by averaging the time courses of the mean ROS movement and ERG magnitude acquired from ten different locations of a retina. Figures 4C2 and 4D2 provide enlarged illustration for the data of the averaged time courses of mean ROS movements and ERG magnitudes in the dashed squares shown in Figures 4C1 and 4D1. The onset time of ROS movements was determined as the corresponding time of the first frame that had a mean ROS movement magnitude larger than the 3- σ threshold (described in the section 2.4). The onset time of ERGs was defined as the time when the ERG amplitude exceeded the 3- σ threshold. As shown by the red triangles in Figure 4C2 and 4D2, the onset times of ROS movement and ERG were 10 ms and 38 ms, respectively, indicating a 28-ms delay for ERG a-wave onset.

4. Discussion

In this study, we investigated the origin of stimulus-evoked ROS movements by superfusing the retina with low-sodium solution and also comparing the onset of ROS movement and ERG a-wave development. Under treatment with low-sodium medium, the ERG responses were significantly prohibited, but the ROS movements were basically preserved in isolated frog retinas. Moreover, the time courses of the mean ROS movement and ERG obtained either from the same location of a retina or from different locations of several retinas presented general concordances in the aspects of the overall waveform profile and peak time. This finding supported our previous hypothesis that the events underlying light-evoked ROS movement are independent from the closure of the cGMP-gated ion channel. The onset times of the ROS movements and ERG a-waves were then compared when the retina was superfused with normal Ringer's medium and we found that the onset of ROS movement occurs ~28 ms earlier than that of the ERG a-wave, indicating an earlier origin of ROS movement than the hyperpolarization of the photoreceptors in the phototransduction cascade. The possible events that result in ROS movement include the upstream, disc-based phototransduction steps, involving the sequential activation of rhodopsin, transducin and cGMP phosphodiesterase (PDE) [16-18].

In one of our earlier studies, *in vivo* OCT experiments with frog retinas revealed that the onset time for the light-evoked IOSs were as early as 4 ms after flash initiation (Fig. 4C in [15]) which was shorter than the 10-ms onset time demonstrated in the present work. Multiple factors are suspected to have caused this difference. Although the ROS movement may be a major contributor to the IOS, it is not necessary that it is the only IOS source. Other factors, including system sensitivity and stimulation efficiency may also be responsible for the difference in onset times. To address these concerns, we are currently constructing a high spatiotemporal resolution ophthalmoscope to pursue an *in vivo* investigation of TRP. Further studies of the TRP mechanism will create a better understanding of the phototransduction processes and provide a valuable biomarker for diagnosing retinal diseases as the TRP reflects a functional response of retina.

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Figure 1.

(A) Schematic diagram of the experimental setup. During the experiment, the frog retina was continuously illuminated by a NIR light source in a transmission mode for image acquisition and superfused with physiological medium. Visible light generated by a fiber-coupled light emitting diode (LED) was collimated and coupled into the light path of the microscope to work as a stimulus. ERG recording was implemented by placing two differential electrodes on the top and bottom of the retina, respectively. (B) The retina was placed in a glass chamber with ROSs facing upward and was obliquely ($\sim 20^\circ$) stimulated by a visible light beam from the top. The direction of ROS movement was defined by the coordinate axes shown in the middle.

Figure 2.

Comparison of ROS movements and ERGs with different superfusion mediums. The imaging and stimulated areas were identical for all three experiments. The retina was perfused consecutively with regular Ringer's medium (control group), low-sodium medium (low Na^+ group) and regular Ringer's medium (control group). The ROS movement magnitude (B1, B2 and B3, respectively) and direction (C1, C2 and C3, respectively) maps were calculated based on the retinal images (A1, A2 and A3, respectively) acquired at time 0.4 s after the onset of the stimulus in each group. The yellow regions in A1, A2 and A3 represent the stimulus pattern projected on the retina. (D) Time courses of the mean ROS movement acquired from the control group (D1), low-sodium group (D2) and control group (D3), respectively. (E) Time courses of the ERG acquired from the control group (E1), low-

sodium group (E2) and control group (E3), respectively. Shaded areas in D and E represent stimulation periods.

Figure 3.

Averaged mean ROS movement magnitudes (A) and ERGs (B) of control (A1 and B1), low sodium (A2 and B2) and the control group (A3 and B3), obtained from six frog retinas. Each trace is the mean and the accompanied gray area is the standard deviation of the data from six retinas. Shaded areas show stimulation periods.

Figure 4.

(A) Representative ROS image acquired at a frame speed of 500 fps. The yellow area shows the stimulation pattern projected on the retina. (B) Representative ROS movement magnitude maps acquired at time 0.2 s before (B1), and 0 s (B2), 0.2 s (B3), 0.4 s (B4), 0.6 s (B5) and 0.8 s (B6) after the onset of the stimulus, respectively. Time courses of the average and standard deviation (gray area) of the mean ROS movement magnitudes (C1) and ERGs (D1) obtained from ten different positions of the retina. C2 and D2 are enlarged pictures of the dashed squares shown in C1 and D1, respectively. The sampling rates for C2 and D2 was 500 Hz. The red triangles in C2 and D2 represent the onset times of ROS movements and ERG a-waves determined by the 3-8 threshold. Shaded areas in C and D demonstrate the stimulation periods.