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Patch Clamp Recordings of Retinal Bipolar Cells in Response to Extracellular Electrical Stimulation in Wholemout Mouse Retina

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Abstract

Retinitis pigmentosa is a family of inherited retinal diseases identified by the degeneration of photoreceptors, which leads to blindness. In efforts to restore vision lost to retinitis pigmentosa, retinal prostheses have been developed to generate visual percepts by electrically stimulating the surviving retinal bipolar and ganglion cells. The response of retinal ganglion cells to electrical stimulation has been characterized through direct measurement. However, the response of bipolar cells has only been inferred by measuring ganglion cell activity. This investigation reports on a novel tissue preparation technique facilitating bipolar cell patch clamp recordings in wholemount retina. We find that bipolar cells respond to extracellular electrical stimuli with time-locked voltage spike depolarizations, which are likely mediated by voltage-gated calcium channels.

I. Introduction

Retinitis pigmentosa (RP) is a family of blinding diseases that result in the degeneration of photoreceptors. Approximately 30% of retinal ganglion cells (RGC) and 80% of bipolar cells (BC) remain after the degeneration [1]. Several groups have developed retinal prostheses that use microelectrode arrays to electrically stimulate the remaining retinal neurons [2]. These devices have been approved for use in humans. *In vitro* retina studies reveal that RGCs respond to electrical stimulation with both a direct response time-locked within a millisecond of the stimulus as well as an indirect response, delayed by 8–60ms [3], originating from inner retinal neurons that synaptically drive RGCs. The direct RGC response can be isolated with short (<0.4ms) pulse widths while the indirect response is isolated through the application of long (>25ms) pulse widths [4]. Stimuli with pulse widths falling between these two extremes activate both RGCs and BCs. Additionally, stimuli capable of directly activating RGCs also have the potential to antidromically activate peripheral RGCs by stimulation of axons passing beneath the electrode [4]. In patients, the antidromic activation is perceived as a streak [5]. However, indirect activation does not activate underlying axons thereby conferring an activation of RGCs localized with respect to the electrode [4].

This report describes a novel photoreceptor peeling technique that for the first time allows direct measurement of bipolar cell responses to epiretinal stimulation. Traditionally, BCs

have been shown to transmit visual information from photoreceptors via graded membrane potential changes in proportion to the excitatory input. However, recent *in vivo* and *in vitro* studies have demonstrated that BCs also exhibit voltage spikes mediated by voltage-gated calcium channels to convey visual signals [6–10]. The spikes are suggested to be a mechanism that can quickly fuse vesicles to the presynaptic membrane terminal. Additionally, the spikes may initiate temporal coding in the retina, a step before the action potentials generated by RGCs [10]. With these signaling mechanisms available, it is critical to understand the manner of excitation seen in the BCs when electrical stimulation is applied, so that we can make well-informed decisions about the best stimulus paradigms for retinal prostheses. Thus, this investigation uses patch clamp to provide direct measurement of BC activity in response to epiretinal electrical stimulation in wholemount retina.

I. Procedures

A. Tissue Preparation

Wild-type Tg(Gng13-EGFP)GI206Gsat mice (n=4) 6–20 weeks of age, in a C57BL/6J background strain, that express EGFP in ON-type BCs were anaesthetized with ketamine/xylazine before cervical dislocation as approved by the IACUC at the University of Southern California [11]. Eyes were enucleated and placed in 30°C sodium bicarbonate-buffered Ames' media (Sigma, Louis, MO) (pH = 7.4, 280mOsm) oxygenated with 95% O₂, 5% CO₂. After the retina was isolated from the sclera, photoreceptor outer segments and somas were removed by placing a quadrant of the excised retina photoreceptor-side down on VWR 415 filter paper (VWR, Visalia, CA) while in solution. The filter paper-containing retina was then held filter paper-side down on paper towel while applying oxygenated Ames' solution atop the retina to force the photoreceptors to adhere to the filter paper. The retina was then placed back into Ames' solution where it was separated from the filter paper with forceps. Care was taken to only touch the extreme perimeter of the retina. The process was performed approximately 10 times in order to remove virtually all photoreceptors somas except the inner most two layers. Successful removal of the photoreceptors was confirmed by viewing the retina under infrared light (780nm) and resolving EGFP-labeled BCs (Fig. 1B). The retina was then mounted RGC-side down on a custom microelectrode array (MEA) and perfused with oxygenated Ames' media.

B. Live Dead Assay

Following photoreceptor peeling, the retina was mounted RGC-side down on a microelectrode array, held down by a nylon membrane, and left undisturbed for 30 minutes while being perfused with the oxygenated Ames' sodium bicarbonate. 5µM calcein blue-AM ester and 1µM ethidium homodimer-1 were applied as the live and dead assays, respectively (Sigma, Louis, MO). The assays were incubated for 15 minutes on the uncovered tissue before resuming the perfusate to wash out the assays and preserve retinal health. Fluorescence imaging was performed to determine the results of the live-dead assay in addition to colocalization of EGFP in the ON-type retinal BCs. Imaging was performed in both the inner nuclear layer (INL) and outer nuclear layer (ONL).

C. Electrophysiology

Voltage clamp recordings were used to collect I–V curves for BCs. Cells were held at -50mV and stepped to test voltages ranging from -80mV to 60mV in increments of 10mV . Each step was held for 200ms . I–V curves were created by analyzing the sustained current response.

Current clamp recordings were used for the remainder of the experimentation. While the holding current was 0pA , current injections of -10pA to 20pA in steps of 2pA were delivered through the pipette for 6 seconds. Oscillation amplitudes and frequencies were obtained by detrending the response with a 7th-order polynomial and thresholding peaks above the rms-noise of the detrended signal. Membrane potentials were estimated from the average of the membrane potential during the stimulus.

Electrophysiological signals were collected using an EPC9 Patch Clamp Amplifier (HEKA, Bellmore, New York) under the control of PatchMaster software. Patch clamp pipettes were pulled from 1.2mm outer diameter borosilicate glass (Sutter Instruments, Novato, CA) with resistance $6\text{--}14\text{M}\Omega$. Patch pipettes were filled with internal solution containing the following components (mM): 120 K-Gluconate , 10 NaCl , 3 ATP-Mg , 0.3 GTP-Na , 10 HEPES , 0.5 EGTA , $10\text{ Phosphocreatine Disodium Hydrate}$, adjusted to $\text{pH}=7.3$ with 1M KOH (Sigma, Louis, MO) and an osmolality of 270mOsm . All recorded signals were corrected for a liquid junction potential measured at 14mV using previously described methods [12].

D. Extracellular Stimulation

Epiretinal electrical stimulation was delivered through $200\text{-}\mu\text{m}$ diameter indium tin-oxide electrodes recessed $5\mu\text{m}$. Stimulation currents were charge-balanced, biphasic, cathodic-first current pulses. The cathodic phase pulse durations applied were 25- , 50- , and 100-ms . Stimulus amplitudes ranged from $0.2\mu\text{A}$ to $2\mu\text{A}$ in $0.2\mu\text{A}$ increments. Each pulse was presented 10 times in a 0.33Hz pulse train and the order of the pulse trains was randomized.

II. Results

A. Photoreceptor Peeling

Filter paper effectively removed photoreceptors outer segments and somas from the retina. Prior to peeling, photoreceptors obscured the details of the ON-type BCs when viewed subretinally (Fig. 1A). After peeling, EGFP fluorescence in ON-type BCs could be visualized (Fig. 1B) Photoreceptor removal also facilitated patch clamp pipette access to the BCs. Some areas of the retina contained fewer photoreceptor somas than others, but no distinct pattern was evident. The center of the retinal section was used for all experiments as this area was never handled during the process of removing the OS.

A concern with mechanically peeling away photoreceptors is that it may damage BCs or alter their endogenous response patterns. To address this issue we performed a live-dead assay on the remaining neurons with calcein blue-AM and ethidium homodimer-1 (Fig. 2). Though the assays did not penetrate deep into the retina, Fig. 2Ac illustrates that the

majority of remaining cells in the INL have calcein-blue fluorescence, which is indicative of intact cell membranes. A minority of cells have compromised membranes as shown by the few cells labelled with ethidium homodimer-1 (Fig. 2Ad). Importantly, there is strong colocalization of the calcein blue-AM with the EGFP fluorescence from ON-type bipolar cells as indicated by the asterisks in Fig. 2Ab–c. Neuronal survival is observed as well in the ONL layer (Fig. 2B) where many cells are labelled with the calcein blue-AM (Fig. 2Bc). EGFP fluorescence (Fig. 2Bb) does not show BCs somas when the image plane is focused in the ONL.

B. Electrophysiological Measurements

We collected I–V curves to assess whether BCs maintained characteristic electrical activity in voltage clamp. Command potentials were stepped from -80mV to $+60\text{mV}$ in 10mV increments from a -50mV holding potential. Fig. 3 illustrates that the I–V curves obtained are comparable to I–V curves measured from bipolar cells in traditional slice preparations in both the current magnitude and outward rectifying current onset [13]. Outward currents (positive value) initiate at approximately -40mV , and current amplitudes rise approximately linearly peaking as high as 1000pA . In some BCs, the I–V curve slope increased, then decreased, for potentials greater than 0mV (data not shown), likely the result of calcium-dependent potassium channels [14].

In current clamp mode, we injected current into BCs ($n=14$) and measured the resultant membrane potential changes. Fig. 4A shows a representative ON-type BC response to current injections. Only a few current step responses are shown for clarity. The negative current steps caused the membrane potential to hyperpolarize while simultaneously decreasing the amplitude of the membrane oscillations. Positive current steps increased the amplitude of the oscillations, peaking at $\pm 6.6\text{mV}$ for which the membrane potential baseline was -39mV (Fig. 4B). Above a membrane potential of -39mV , the oscillation amplitude diminished. These results support previous findings that BC spiking is observed within a membrane potential window [9]. Fig. 4C shows the dominant frequency component against membrane potential obtained from the Fourier transform of the voltage response. Oscillation frequency is maximal at a membrane potential of -36mV , and appears to loosely correlate with the oscillation amplitude.

C. Electrical Stimulation Responses

BCs respond to electrical stimulation with voltage spikes time-locked with stimulus presentation. Fig. 5A shows the response of a BC to ten 25-ms $0.8\mu\text{A}$ current pulses delivered at 0.33Hz (stimulus times indicated by tick marks). Through a $200\text{-}\mu\text{m}$ diameter electrode this equates to a $63.7\mu\text{C}/\text{cm}^2$ charge density for each pulse. Baseline spikes were also observed independent from stimuli. To compare the kinetics of baseline and the evoked spikes, we removed the 25-ms , $0.8\mu\text{A}$ stimulus artifact from the evoked responses by subtracting the mean recorded artifact from stimulus presentations that did not evoke a spike. We averaged spikes with similar rise times and compared this with the mean baseline spike that is superimposed in Fig. 5B such that the stimulus in the evoked trace begins at 200ms . The average spike amplitudes were 20.6mV and 17.1mV for the baseline and evoked spike, respectively. Full-width at half-maximum was 37ms and 27ms , respectively.

Each displays a refractory period of approximately 130ms. The evoked spike reached its peak amplitude 35ms after the stimulus onset. Rise times for spikes evoked by this stimulus ranged from 13–40ms. This rise-time range coincides well with the 8–60ms indirect response latency observed in RGCs [3]. The baseline spike appears to have a slower rise in amplitude before spiking when compared to the evoked spike. Evoked spikes may not exhibit the gradual depolarization because the electrical stimulus quickly brings the membrane potential to the threshold for generating spikes.

After observing reproducible time-locked spike responses in BCs, we tested various stimulus amplitudes to determine a stimulus threshold. Fig. 6 shows the resultant dose-response curve for a cell in response to 25-ms stimulation. The probability of evoking a response increased as the stimulus amplitude increased. We defined threshold as the lowest current amplitude necessary to elicit a spike 50% of time. Extending this procedure, we measured BC thresholds ($n = 7$) in response to 25-, 50- and 100-ms pulses (Fig. 7). As expected, threshold current amplitudes decreased as the pulse width was lengthened.

III. Conclusion

The photoreceptor peel technique developed in this study allowed us to directly measure the response of retinal BCs to extracellular electrical stimulation in the wholemount retina via patch clamp. A wholemount retina preparation is significant because it better mimics the operational environment of the retinal prosthesis in comparison to a slice or dissociated cell culture preparation. Our findings demonstrate that BCs respond to extracellular electrical stimulation with time-locked voltage spikes. The time course of the spike response makes it a good candidate for mediating the long-latency indirect response observed in RGCs.

Traditionally, BCs are thought to only produce graded potentials in proportion to light stimulus intensity, but previous works have demonstrated that light stimuli can evoke voltage spikes mediated by voltage-gated calcium channels [6–10]. Patch clamp recordings of light-evoked spike responses demonstrate kinetics similar to those elicited by electrical stimulation in this study [7]. Taken together, it is a likely conclusion that voltage spikes elicited by electrical stimulation are also mediated by voltage-gated calcium channels.

BCs contain L- and T-type voltage gated calcium channels whose expression and morphological distribution vary across BC subclasses [15]. Understanding the contribution of each type in the formation of the BC response to electrical stimulation may lead to novel stimulus paradigms in retinal prostheses that more effectively activate BCs.

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Figure 1. Photoreceptor peeling allows visualization of BCs. **A** Observed EGFP fluorescence from ON-BCs viewed through intact photoreceptors. **B** View of EGFP fluorescence from ON-BCs after photoreceptor peeling. Scale bar = 10 μ m.

Figure 2.

Live-dead assay of the retina after performing photoreceptor peel technique. **A** View of the INL. **Aa** Retina under infrared illumination, **Ab** EGFP fluorescence in ON-type BCs. **Ac** Live-assay labelled cells. **Ad** Dead-assay labelled cells. **Ae** Composite image. **Ba–e** View of ONL with same labeling as in **Aa–e**, refers to ONL. Scale bar in **Aa** = 10 μ m.

Figure 3.

Bipolar cell I-V curves recorded in wholemount retina. **A** Bipolar cell currents in response to voltage steps from -80mV to 60mV in steps of 10mV from a -50mV holding potential. **B** Current vs. voltage plot. Current measured 150ms after stimulus onset.

Figure 4.

ON-type BC response to current injections applied on the soma through a patch pipette.

Current was stepped from -10 to 20 pA in 2 pA increments. **A** Representative voltage response to current steps applied from 2 to 8 seconds. **B** Binned oscillation amplitude and **C** binned dominant frequency component of the oscillations as a function of membrane potential. Error bars represent standard deviations. ($n=14$).

Figure 5.

A Bipolar cell response to 25-ms, 0.8 μ A stimulation delivered at 0.33Hz (ticks mark stimulus presentation) **B** Averaged bipolar cell voltage spikes from baseline activity (dotted) and stimulus-evoked spike (solid) from a 25-ms 0.8 μ A stimulus. Stimulus artifact at 200ms was removed from the evoked spike trace.

Figure 6.

Dose-response curve of a bipolar cell to 25-ms cathodic first electrical stimulation.

Threshold is defined as the stimulus amplitude required to elicit a response in 50% of stimulus presentations.

Figure 7. Stimulation thresholds for ON-type bipolar cells ($n=7$) in response to epiretinal extracellular stimulation. Stimuli pulse widths are 25-, 50- and 100-ms charge-balanced, cathodic first current pulses. Error bars represent standard deviation.