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A Comparison of Optical and Electrophysiological Methods for Recording Retinal Ganglion Cells during Electrical Stimulation

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Abstract

Purpose/Aim—To compare the efficacy of optical techniques with electrophysiological recordings for mapping retinal activity in response to electrical stimulation.

Materials and Methods—Whole cell patch clamp, Ca²⁺ imaging (Fluo-4-AM), and Na⁺ imaging (CoroNa Green-AM) techniques were used to detect responses of neurons from mouse and salamander retina to electrical stimulation.

Results—Synaptic currents were observed in 23% of retinal ganglion cells (RGCs), indicating presynaptic Ca²⁺ increases in the inner plexiform layer (IPL). Modest depolarization with 20–30 mM K⁺ consistently evoked Ca²⁺ responses measured with Fluo4, but Ca²⁺ responses were almost never evoked by epiretinal stimulation. In salamander retina, responses were seen in the inner nuclear layer (INL) and IPL. In mouse retina, responses were also sometimes seen in the outer plexiform layer (OPL). OPL responses showed a longer latency than IPL responses, suggesting that outer retinal circuits do not trigger synaptic responses of RGCs. Simultaneous Ca²⁺ imaging and electrophysiological recording of synaptic currents confirmed that Fluo4-loaded retinas remained responsive to stimulation. Epiretinal stimulation evoked action potentials in 67% of RGCs. CoroNa Green detected Na⁺ changes stimulated by 20 mM K⁺, but epiretinal stimulation did not evoke detectable Na⁺ responses. Simultaneous imaging and electrophysiological recording confirmed the health of CoroNa Green-loaded retinas. We confirmed stimulation efficacy by simultaneously recording Na⁺ changes and electrophysiological responses.

Conclusions—These data demonstrate that electrophysiological recordings show greater sensitivity than Na⁺ or Ca²⁺ imaging in response to electrical stimulation. The paucity of Ca²⁺ responses is consistent with limited risk for Ca²⁺-mediated cell damage during electrical stimulation.

Keywords

Retina; Electrical stimulation; Whole cell recording; Calcium imaging; Sodium imaging

INTRODUCTION

Permanent loss of photoreceptors results in vision loss and occurs in many types of degenerative diseases, such as retinitis pigmentosa, Usher syndrome, and age-related macular degeneration.^{1,2} Fortunately, large numbers of bipolar and retinal ganglion cells (RGCs) survive and remain relatively healthy in those diseases.³ Damaged photoreceptors can therefore be bypassed by electrically stimulating the surviving inner retinal neurons with a prosthetic device implanted on the retinal surface.^{1,2,4-9}

To design more effective and efficient retinal prostheses, one needs to understand the retinal neurons and circuits engaged by stimulation. Epiretinal electric stimulation can generate action potentials by directly stimulating ganglion cells but can also indirectly activate synaptic currents in ganglion cells by stimulating presynaptic neurons, including bipolar and amacrine cells.¹⁰⁻¹⁴ One approach to analysing the different cell types and retinal circuits engaged by electrical stimulation is to obtain electrophysiological recordings from individual neurons. Another approach is to obtain extracellular recordings from multiple cells simultaneously.¹⁵ However, this approach cannot be used for recording the activity of non-spiking retinal neurons (e.g. bipolar cells). We therefore investigated two optical techniques for analysing the activity of retinal neurons during epiretinal stimulation using a slice preparation that allows visualization of all different types of retinal neurons simultaneously. In one approach, we used a Ca^{2+} -sensitive fluorescent dye, Fluo4, to visualize intracellular Ca^{2+} changes with a confocal microscope. Stimulation of ganglion cells often evokes synaptic currents that originate with inputs from bipolar cells, amacrine cells, and possibly photoreceptor cells.^{7,12} Because synaptic transmission is triggered by Ca^{2+} influx into presynaptic terminals, the presence of synaptic currents in ganglion cells suggests that epiretinal stimulation necessarily evokes Ca^{2+} increases in presynaptic neurons. In addition, stimulation might also activate other voltage-gated Ca^{2+} channels that do not participate in synaptic release from retinal neurons.^{16,17} The high surface to volume ratio in small dendrites combined with the large trans-membrane Ca^{2+} gradient allows the visualization of Ca^{2+} changes even within small dendrites.^{18,19} We therefore hypothesized that examining retinal slices with Ca^{2+} imaging would be a good way to visualize the activity of many cells simultaneously.

CoroNa Green has been used to detect intracellular Na^+ changes in small dendritic processes evoked by glutamate receptor activation.²⁰ Sodium channels are concentrated in a band on the axon ~40 microns from the cell body²¹ and we hoped that action potentials or other depolarizing activity in ganglion cells and other retinal neurons might produce sufficiently large local Na^+ changes to visualize individual cell processes.

We evaluated both salamander and mouse retina to examine the efficacy of these two optical techniques in mapping retinal neuronal activity in response to electrical stimulation, comparing them to recordings from single cells using whole cell electrophysiological recording techniques. Epiretinal stimulation only rarely evoked Na^+ or Ca^{2+} responses. By simultaneous imaging and electrophysiological recordings, we confirmed that the rarity of Na^+ or Ca^{2+} responses was not due to compromised tissue, but was more likely due to the limited size of Na^+ or Ca^{2+} changes. Thus, electrophysiological recording from RGCs shows a far greater sensitivity than Na^+ or Ca^{2+} imaging techniques and is superior for mapping RGC responses to electrical stimulation. In mouse retina, we observed Ca^{2+} responses in the outer plexiform layer (OPL) but these responses showed a longer latency than responses in the inner plexiform layer (IPL), suggesting that they are unlikely to be responsible for triggering synaptic responses in RGCs. The small size and number of Ca^{2+} responses evoked by retinal stimulation is consistent with a limited risk for Ca^{2+} -mediated cell damage during electrical stimulation.

MATERIALS AND METHODS

Animal Use

Mice (*Mus musculus*, C57BL6J) (Jackson Laboratory, Bar Harbor, ME, USA) and aquatic tiger salamanders (*Ambystoma tigrinum*, Kons Scientific, Germantown, WI, USA) were handled humanely in accordance with the U.S. Public Health Service policy on Human Care and Use of Laboratory Animals. Protocols were approved by the Veterans Administration and University of Nebraska Medical Center Animal Care and Use Committees.

Mouse Retina

Techniques for preparing slices and flatmount retina preparations have been described previously.²² Briefly, mice were euthanized by CO₂ inhalation. The eye was then rapidly enucleated and the retina was isolated-vitreous surface down - on a piece of nitrocellulose filter paper (Type AAWP, 0.8 µm pores, Millipore, Bedford, MA, USA) under cold Ames' medium. For flatmount retina experiments, retinas were isolated on a 10 × 10 mm piece of filter paper with a 4 mm diameter hole cut into its centre. For retinal slice experiments, slices (125 µm) were cut with a razor blade tissue chopper (Stoelting, Wood Dale, IL, USA) and rotated in the recording chamber to view the retinal layers. Preparations were viewed on an upright fixed stage microscope (Olympus BHWI, Tokyo, Japan or Nikon E600 FN, Tokyo, Japan). Tissue was superfused at room temperature or 37°C at a rate of ~1 mL min⁻¹ with Ames' medium that was continuously bubbled with 95% O₂ and 5% CO₂.

Salamander Retina

Salamanders were decapitated with heavy shears and the brain and spinal cord rapidly pithed. After enucleation, the anterior segment of the eye, including the lens, was removed. Retinal slices were prepared with techniques similar to those described for mice above and superfused at ~1 mL min⁻¹ with a Ringer solution containing (in mM): 101 NaCl, 22 NaHCO₃, 2.5 KCl, 2 CaCl₂, 0.5 MgCl₂, and 9 glucose. The tissue was continuously bubbled with 100% O₂.

Electrophysiology

Whole cell recordings were obtained using 10–15 MΩ patch electrodes fabricated from borosilicate glass (1.2 mm o.d. 0.95 mm i.d. with internal filament, World Precision Instruments, Sarasota, FL, USA) on a PP-830 micropipette puller (Narishige USA, East Meadow, NY, USA). Ganglion cells were voltage clamped at a steady holding potential of –70 mV (mice) or –60 mV (salamander). The pipette solution for whole cell voltage-clamp recordings from mouse RGCs contained (in mM): 125 cesium gluconate, 10 TEACl, 10 Hepes, 3 EGTA, 1 ATP, 0.5 GTP, 3 MgCl₂, and 1 CaCl₂ (pH 7.2). The pipette solution for whole cell voltage-clamp recording from salamander RGCs contained (in mM): 94 cesium gluconate, 9.4 TEACl, 1.9 MgCl₂, 9.4 MgATP, 0.5 GTP, 5 EGTA, and 32.9 Hepes (pH 7.2). The use of TEA blocked K⁺ channels and thus helped to improve space clamp, diminishing the likelihood of repetitive spiking in voltage-clamped cells. Currents were acquired using an Axopatch 200B amplifier (Axon Instruments, Molecular Devices, Sunnyvale, CA) and Digidata 1440A or 1322A interface plus pCLAMP 9.2 software (Axon Instruments, Sunnyvale, CA).

Imaging

Confocal images were acquired using a laser confocal scanhead (Perkin-Elmer Ultraview LCI, Waltham, Massachusetts) equipped with a cooled CCD camera (Orca ER, Hamamatsu Photonics, Hamamatsu City, Japan) and mounted to a fixed stage upright microscope (Nikon E600 FN, Nikon Corporation, Tokyo, Japan). Excitation and emission were controlled by a

Sutter Lambda 10-2 filter wheel and controller. Images were acquired and analysed using UltraView Imaging Suite software (Waltham, Massachusetts). The objective (60 $\times$; 1.0 NA) was controlled using a Z-axis controller (E662 Physik Instrumente, Karlsruhe, Germany) to acquire a series of z-slices (1 μ m steps) at each time point. Frame duration ranged from 57 to 200 ms and each z-stack was acquired at 10 s intervals.

For Ca²⁺ imaging in salamander, retinal slices were incubated with 10 μ m Fluo-4-AM + 5 μ m pluronic F-127 (Invitrogen, Carlsbad, CA), for 45 min in the dark, followed by an additional incubation in Fluo-4-AM alone for 1.5 h (5°C). For Ca²⁺ imaging in mouse retina, slices were incubated with 0.5 mL of 10 μ m Fluo-4-AM in Hibernate A medium (Brainbits LLC, Springfield, IL) for 2 h at room temperature in darkness. For Na⁺ imaging, salamander retinal slices were incubated with 10 μ m CoroNa Green (Invitrogen, Carlsbad, CA) for 120 min in the dark. Images were analysed by comparing pre- and post-stimulation images using UltraView Imaging Suite software (Perkin-Elmer, Waltham, Massachusetts) and Metamorph (Universal Imaging, Molecular Devices, Sunnyvale, CA).

In some experiments, salamander retinal cells were depolarized by bath application of solutions containing 20 mM KCl and mouse retinal cells by bath application of 30 mM KCl. These solutions were formulated by replacing equimolar amounts of NaCl with KCl in the amphibian superfusate described above. Application of 20 mM K⁺ depolarizes light-adapted salamander rods to $\sim$ -35 mV.²³ Similarly, 30 mM K⁺ depolarizes mouse rods to $\sim$ -35 mV.²²

Electrical Stimulation

Tungsten electrodes with resistance of 50–100 k Ω (FHC, Bowdoin, ME) were positioned near the vitreal surface of the retina. This particular resistance was chosen because it resembles the resistance of stimulating electrodes in a 3200 electrode designed by the Naval Research Laboratory in a new epiretinal prosthesis. This electrode array is being examined in our laboratory for future implantation in animals and humans. It also resembles other prostheses such as the one designed by Second Sight (Argus I) in which electrode impedance varies between 13–40 k Ω .²⁴ During electrophysiological recordings, stimulating electrodes were positioned $\sim$ 30 μ m from the recorded cell. The retina was stimulated with monophasic cathodic current pulses (A-M Systems, Model 2100, Sequim, WA or Master-8, A.M.P. Instruments, Jerusalem, Israel).

Cell Identification

For electrophysiological experiments, cells were classified as ganglion cells based on their presence in the ganglion cell layer and the presence of prominent fast inward Na⁺ currents. Many cells in the mouse RGC layer (59%) are displaced amacrine cells.²⁵ It is thus likely that some of the cells we classified as ganglion cells were displaced amacrine cells. ON and OFF cell subtypes were classified by the light responses evoked by a bright white LED.²⁶

Statistics

Numerical values are given as mean $\pm$ SD. Statistical significance was determined with Student's *t*-test using paired and unpaired samples. The criterion for statistical significance was chosen to be $p < 0.05$.

Unless otherwise stated, chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

RESULTS

Electrophysiology

Epiretinal stimulation can evoke both action potentials and synaptic currents in RGCs from salamander and mouse retina.^{7,12} When RGCs are voltage clamped, an electrical stimulus pulse can evoke action potentials that result in large, transient inward currents due to the activation of fast voltage-gated Na⁺ channels. Examples are provided in Figure 1, which shows that brief cathodic electrical stimulation pulses evoked spikes in mouse and salamander RGCs.

In flatmount mouse retina, spikes were evoked in 75% (27/36 cells) of RGCs. Using a tungsten stimulating electrode ($R = 50\text{--}100\text{ k}\Omega$) positioned less than 30 μm from the voltage-clamped cell, the current threshold needed to evoke spikes in ganglion cells from flat-mount mouse retina averaged $12.5 \pm 2.1\text{ }\mu\text{A}$ ($n = 27$) at 1 ms duration (charge density of $0.025 \pm 0.004\text{ mC/cm}^2$). Spikes were also evoked in 69% (9/13 cells) of mouse RGCs in retinal slices. Spike threshold in RGCs from slice preparations averaged $14.1 \pm 1.7\text{ }\mu\text{A}$ ($n = 9$), slightly higher than that in flatmount retina.

In addition to evoking spikes, electrical stimulation also evoked synaptic currents in RGCs. The predicted value for E_{Cl} in these experiments was $\sim -20\text{ mV}$ and so the activation of GABA or glycine currents should stimulate an inward current at the holding potential of -70 mV . Previous studies indicate that these currents are largely due to release of GABA and glycine from amacrine cells.^{7,12} Epiretinal stimulation triggers the release of GABA and glycine by stimulating release of glutamate from bipolar and perhaps photoreceptor cells.^{7,12} Synaptic currents were observed in 36% (13/36) of mouse RGCs in flatmount retina and 23% (3/13) of mouse RGCs in retinal slices. The threshold for evoked synaptic currents in RGCs from flatmount mouse retina averaged $6.4 \pm 1.2\text{ }\mu\text{A}$ ($n = 13$) with 30 ms duration, slightly lower than that from mouse retinal slices ($8.3 \pm 2.0\text{ }\mu\text{A}$, $n = 3$, duration 30 ms). Because longer duration stimuli ($>5\text{ ms}$) were required to evoke sustained synaptically mediated currents, the average stimulation charge density (0.36 mC/cm^2) needed to evoke sustained responses ($n = 13$) was higher than the charge density needed for action potentials (0.11 mC/cm^2 , duration 10 ms, $n = 27$, $p < 0.005$).

We also recorded from RGCs in salamander retina. 67% (8/12 cells) of RGCs in flatmount retina and 68% (13/19 cells) of RGCs in retinal slices exhibited spikes. Spike threshold in RGCs from flatmount preparations averaged $10.2 \pm 1.9\text{ }\mu\text{A}$ ($n = 8$) with 1 ms duration. Spike threshold in RGCs from slice preparations was similar ($p = 0.15$), averaging $11.4 \pm 1.7\text{ }\mu\text{A}$ ($n = 13$) with 1 ms duration. The spike threshold in RGCs from flatmount salamander retina was significantly lower than that of RGCs in flatmount mouse retina ($p = 0.01$). The spike threshold of RGCs in salamander retinal slices was also significantly lower than that of RGCs in mouse retinal slices ($p = 0.002$). Synaptic currents were observed in 41% (5/12 cells) of salamander RGCs in flatmount retina and 32% (6/19 cells) of salamander RGCs in retinal slices. The threshold for evoked synaptic currents averaged $6.2 \pm 1.3\text{ }\mu\text{A}$ (30 ms, $n = 5$) in flatmount retina and $7.6 \pm 1.5\text{ }\mu\text{A}$ ($n = 6$, 30 ms duration, $p = 0.12$) in retinal slices. These did not differ significantly from threshold values in mouse retina.

Ca²⁺ Imaging

Because synaptic release is triggered by Ca²⁺ influx into presynaptic terminals, the ability of epiretinal stimulation to evoke synaptically-mediated currents in many RGCs from both mouse and salamander retina indicates the presence of presynaptic Ca²⁺ increases in the IPL and perhaps OPL.^{7,12} To measure intracellular Ca²⁺ changes, we loaded retinal slices from mouse and salamander by incubating them in the AM ester of the Ca²⁺-sensitive dye, Fluo4 (10 μM). Figure 2A shows a confocal stack of a Fluo4-loaded slice from a salamander

retina; Figure 2B shows a mouse retinal slice. In both preparations, photoreceptors were particularly well-loaded by this technique, perhaps because of the large surface area of their outer segments. However, cell bodies in the inner nuclear layer (INL) and puncta in the IPL could also be visualized. Bath application of 20 mM K^+ to salamander retina or 30 mM K^+ to mouse retina depolarizes rod photoreceptors by ~ 10 mV from ~ -50 mV to ~ -35 mV.^{22,23} Depolarization of the retina with these high K^+ solutions stimulated detectable Ca^{2+} changes throughout the inner and outer retina of both species (Figure 2). For example, the graphs in Figure 2 show Ca^{2+} changes in rod somas (region 1, Figure 2A and C), amacrine cell bodies in the INL (region 2, Figure 2A and C), and synaptic puncta within the IPL (regions 3 and 4, Figure 2A; regions 3–5, Figure 2C).

Although these modest concentrations of K^+ produced detectable changes in almost every cell, mouse retinal slices loaded with Fluo4 rarely showed Ca^{2+} increases in response to electrical stimulation. We typically used 100 ms test pulses, increasing the stimulus amplitude to levels that electrophysiological experiments indicated were supra-threshold for spikes and synaptic currents (15–20 μ A). Higher currents exceeded the water window (i.e. the amount of current that induces electrolysis of water) for the stimulating electrode. In experiments with over 100 slices, Ca^{2+} responses were observed very rarely: ONL, 0 cells/101 retinal slices; OPL, 16 cells; INL, 3 cells; IPL, 9 cells; RGC, 0 cells). Figure 3 shows the slice with the most stimulus-evoked activity of any that we studied. In this slice, Ca^{2+} increases were evoked in the INL and IPL by electrical stimulation. Panel A shows a single confocal section of the retina loaded with Fluo4. Panel B shows the “difference” image (subtracting the image before stimulation from the image at the end of a 100 ms cathodic current pulse). Three cell bodies in the INL and two puncta in the IPL were activated by stimulation. Fluo4 fluorescence changes measured in these regions are plotted in panel C. Responses rose rapidly to a peak upon stimulation and then declined rather slowly over the next few seconds. The slow decline observed in this and other slices indicates that responses were not too rapid to be observed with image acquisition rates of 57–200 ms/frame.

The presence of detectable Ca^{2+} changes in the OPL has been cited as evidence that photoreceptor inputs may contribute to shaping the synaptic responses observed in RGCs.¹² To test this hypothesis, we compared the latency to the beginning of Ca^{2+} responses in the IPL and OPL. In all but one case, responses in the IPL were observed by the first image after stimulation (122 ± 68 ms, $n = 9$). By contrast, responses in the OPL showed a significantly longer latency (251 ± 87 ms; $n = 16$, $p < 0.0019$, Figure 4). Although we observed responses from only a few cells out of the many thousands of cells contained within 101 retinal slices, the finding that responses in the OPL exhibited a significantly longer latency than those in the IPL is inconsistent with the hypothesis that Ca^{2+} changes in the OPL were responsible for triggering synaptic responses in the IPL. Furthermore, as described below, electrical stimulation did not evoke detectable Ca^{2+} responses in the OPL in salamander retina are not necessary for observing synaptic responses electrophysiologically or detecting Ca^{2+} increases in the IPL and INL.

We were concerned about the possibility that the dye-loading procedure may have had deleterious effects on the health of the tissue and thus limited tissue responsiveness. We therefore tested a shorter loading time of 45 min at both room temperature and 37°C, but did not observe a greater frequency of Ca^{2+} responses. Dye loading was substantially diminished by incubation at 37°C, perhaps because of the greater activity of multi-drug resistance transporters.²⁷ We also tested loading for 2 hrs at a colder temperature of 4°C, but once again did not observe a greater frequency of Ca^{2+} responses.²⁷ In addition to different loading protocols, we also compared Ca^{2+} responses evoked in slices that were loaded in the normal way, but superfused at either room temperature or 37°C. Warming the superfusate

did not noticeably increase the frequency of Ca^{2+} responses to electrical stimulation in mouse retinal slices.

We then examined Ca^{2+} responses in salamander retinal slices, reasoning that this preparation is more physiologically robust than mouse retina. As with mouse retina, stimulation evoked only occasional Ca^{2+} increases in salamander retina (Figure 5). One difference between salamander and mouse retina was that increases were not observed in the OPL from salamander retina. Electrically evoked increases were observed in 7 INL cells from 50 slices, 7 puncta in the IPL, and 2 RGCs. Responses in the IPL were detected within the first frame after stimulation (73 ± 42 ms, $n = 7$). Responses in the INL showed a slightly longer latency (147 ± 69 ms, $n = 7$). Figure 5 shows representative responses evoked by stimulation in the INL (region 1) and IPL (region 2). As in the previous figure, we show a single confocal section of the retinal slice, the difference image, and a graph of Fluo4 fluorescence changes as a function of time. Like mouse retina, responses rose rapidly to a peak and then slowly declined.

As a further test of whether the procedure for dye loading substantially impaired the physiological state of retinal neurons, we obtained whole cell recordings from RGCs in Fluo4-loaded salamander slices. RGCs from dye-loaded slices exhibited fast sodium currents and a high input resistance consistent with healthy cells (1.1 ± 0.5 G Ω , $n = 9$). Additionally, synaptic currents were consistently evoked by electrical stimulation in Fluo4-loaded slices. The presence of synaptic currents indicates that epiretinal stimulation activated the influx of Ca^{2+} into presynaptic terminals of many cells, even though intracellular Ca^{2+} changes were rarely seen. Figure 6 shows an example in which we recorded synaptic currents while simultaneously imaging Ca^{2+} changes. In this experiment, electrical stimulation evoked a long-lasting burst of synaptic activity following application of a 100 ms cathodic pulse. The current pulse also stimulated a detectable Ca^{2+} increase in a single cell body from the INL, but changes in the IPL or other cells from the INL were not detected. Ca^{2+} responses were also not detected in the IPL from 5 other slices in which we conducted Ca^{2+} imaging while simultaneously recording synaptic currents. These results indicate that the rarity of Ca^{2+} responses is not due to unresponsive tissue, but is more likely due to the presence of small Ca^{2+} increases that are not readily detected even after using a relatively high affinity dye ($K_d \sim 345$ nm) and moderately fast acquisition times of 57–200 ms/frame (Figure 6).

Na⁺ Imaging

The ability of epiretinal stimulation to evoke depolarizing spikes in the majority of RGCs suggested that it might be possible to map depolarizing activity in a retinal slice using a Na⁺ dye, CoroNa Green. We therefore loaded salamander or mouse retinal slices by incubating them in 10 μM CoroNa Green-AM. As with Fluo4, photoreceptors were more often strongly loaded than other cells (Figure 7). We explored the sensitivity of CoroNa Green to modest changes in membrane potential by bath applying of various concentrations of K⁺ to salamander retina slices. As illustrated in Figure 7, 20 mM K⁺ evoked detectable changes in CoroNa Green fluorescence in all types of retina neurons including bipolar cells, amacrine cells, and RGCs. Similar results were observed in 6 other experiments.

Although modest depolarization with high K⁺ solutions evoked detectable changes throughout the retina, CoroNa Green fluorescence changes were not detected in either salamander (35 slices) or mouse (20) retinal slices following epiretinal stimulation. As with Ca^{2+} dyes, we tested whether the dye loading procedure may have limited retinal responsiveness by obtaining whole cell recordings from RGCs in CoroNa Green-loaded salamander slices. Like control slices that were not loaded with dye, fast Na⁺ currents could be consistently evoked in RGCs by electrical stimulation in CoroNa Green-loaded slices (n

= 7). RGCs from dye-loaded slices also exhibited spontaneous miniature synaptic currents, synaptic currents evoked by epiretinal stimulation, and high input resistance consistent with healthy cells ($1.0 \pm 0.4 \text{ G}\Omega$, $n = 7$). The presence of fast Na^+ currents indicates that epiretinal stimulation activates the influx of Na^+ into RGCs, even though intracellular Na^+ changes were rarely seen. Figure 8 shows an example in which we imaged CoroNa Green fluorescence during epiretinal stimulation that evoked fast Na^+ currents in a voltage-clamped RGC. As with other slices, intracellular Na^+ changes were not detected by CoroNa Green despite the clear evidence for the ability of epiretinal stimulation to activate action potentials (Figure 8). Thus, the inability to detect CoroNa Green fluorescence changes is not due to an absence of responsive cells, but more likely reflects the presence of Na^+ changes that are simply too small to be readily detected using this dye.

DISCUSSION

The effects of epiretinal stimulation on RGCs have been studied in a variety of species, including salamander,⁷ frog,²⁸ chicken,²⁹ rabbit,³⁰ and mouse.³¹ Most of these studies used electrophysiological recording techniques, largely extracellular recording methods, to document neuronal activity in response to electrical stimulation. In this study, we compared three methods for recording the activity of retinal neurons in response to epiretinal electrical stimulation: whole cell voltage-clamp recording, Na^+ imaging, and Ca^{2+} imaging using both mouse and salamander retinas.

We found that electrical stimulation of salamander or mouse RGCs evoked frequent spikes or synaptic currents that could be easily detected with patch clamp techniques. However, we rarely observed detectable changes using Na^+ or Ca^{2+} imaging techniques in response to epiretinal stimulation. This was not due to poor dye loading since both Na^+ and Ca^{2+} sensitive dyes showed good loading. Moreover, modest depolarizing stimulation by bath application of 20 mM K^+ in salamander retina produced detectable changes with both dyes. Application of 30 mM K^+ also produced detectable Ca^{2+} changes in mouse retina. The ability of high K^+ to evoke responses argues against the possibility of excessive dye loading which can potentially reduce responses by supplementing endogenous buffers. The occasional Ca^{2+} responses that were observed showed a relatively slow decay suggesting that image acquisition rates of 57–200 ms/frame were capable of detecting stimulation evoked responses. Another possible explanation for the rarity of responses is that the dyes or loading process may have caused tissue damage. However, this does not appear to be the case since RGCs from dye-loaded retinas exhibited healthy electrophysiological activity.

The absence of detectable sodium responses in the soma or dendrites of RGCs suggests there is not a large influx of sodium into these compartments. Sodium channels are concentrated in a band on the axon about 40 microns from the cell body.²¹ The ability to detect spikes electrophysiologically suggests that sodium channels were not excised by the slicing procedure. However, we did not see Na^+ responses in the nerve fiber layer. One could argue that we may have missed seeing changes localized to sodium bands because they may have been in a different plane of the retina than the confocal section. However, we examined many RGCs using many slices, suggesting that the absence of responses is unlikely to be a consequence of under-sampling. A more likely explanation to the fact that we did not detect any Na^+ responses is that the ion changes evoked by electrical stimulation were simply too small to be readily detected by CoroNa Green.

Although the frequency of synaptic currents observed electrophysiologically suggests that presynaptic Ca^{2+} increases should be present in many cells within the IPL, we saw Ca^{2+} responses in less than one cell per retinal slice. As discussed above, this does not appear to be due to unhealthy or unresponsive tissue, but instead suggests that epiretinal stimulation

evokes only small intracellular Ca^{2+} changes. This is good news for those considering the use of chronic stimulating arrays since excessive Ca^{2+} levels in cells can be cytotoxic. Furthermore, Ca^{2+} changes in mouse retina were most often seen in the OPL and the patient population that is most likely to receive retinal implants have outer retinal degeneration with few remaining healthy photoreceptors.

Although Ca^{2+} responses were rarely observed, the finding that Ca^{2+} changes in the IPL typically preceded those in the OPL argues against the hypothesis that synaptic currents evoked by lengthy stimulation result from the activation of photoreceptor inputs.^{12,18,32} The absence of Ca^{2+} responses in the OPL of salamander retina also indicates that Ca^{2+} increases in the OPL are not necessary for the generation of Ca^{2+} changes in the IPL.

We conclude that electrophysiological techniques are more sensitive and reliable than dye imaging techniques for recording cellular activity during retinal electrical stimulation. However, a limitation to whole cell recording is that the number of cells that can be examined in a single experiment is limited. One possible solution is to record with arrays of multiple extracellular electrodes.^{15,33} Imaging of intrinsic optical signals might also provide a means for imaging the activity of multiple cells simultaneously.³⁴

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FIGURE 1.

Fast action potential currents (spikes) and synaptic currents evoked from mice and salamander retinal ganglion cells by epi-retinal stimulation. (A) Spike (arrow) evoked in a mouse ON ganglion cell from a flatmount retina preparation by injection of cathodic current (1 ms, 12 μ A). (B) Synaptic current (arrow) evoked in a different ON ganglion cell from a flatmount mouse retina preparation (25 ms, 18 μ A). (C) Spike (arrow) evoked in retinal ganglion cell from a salamander retinal slice (25 ms, 10 μ A). (D) Synaptic current (arrow) evoked in retinal ganglion cell from a salamander retinal slice (30 ms, 15 μ A).

FIGURE 2.

Intracellular Ca^{2+} changes evoked by modest depolarizing stimulation. (A) A confocal stack (fifteen 1 μm sections) of a salamander retinal slice loaded with the Ca^{2+} -sensitive dye, Fluo4. (B) Fluo4 fluorescence changes evoked by bath application of 20 mM K^+ which is sufficient to stimulate ~ 10 mV depolarization in rod photoreceptors. Ca^{2+} changes are plotted as a function of time for a rod soma (region 1 in panel A), an amacrine cell soma (region 2), and synaptic puncta within the IPL (regions 3 and 4). (C) A confocal stack (15 sections) of a mouse retinal slice loaded with Fluo4. (D) Fluo4 fluorescence changes evoked by bath application of 30 mM K^+ which is sufficient to stimulate ~ 10 mV depolarization in rod photoreceptors. Ca^{2+} changes are shown for a rod soma (region 1 in panel A), an amacrine cell soma (region 2), and synaptic puncta in the IPL (regions 3–5).

FIGURE 3.

Detection of Ca^{2+} changes in response to epiretinal stimulation in the inner retina of a mouse retinal slice. (A) Single confocal section of a Fluo4-loaded retinal slice showing the IPL and a portion of the INL. (B) Difference image showing Ca^{2+} changes created by subtracting the control image from a post-stimulus image obtained immediately after stimulation (100 ms, 14 μA). (C) Ca^{2+} changes in three cell bodies from the INL and two puncta from the IPL plotted as a function of time. The corresponding regions of interest are denoted in panels A and B.

FIGURE 4.

Latency from initiation of the cathodic stimulus pulse to the beginning of the Ca^{2+} response in the IPL and OPL. Responses in the OPL showed a significantly longer latency than those observed in the IPL ($p < 0.0019$).

FIGURE 5. Detection of Ca^{2+} changes in response to epiretinal stimulation in a salamander retinal slice. (A) Single confocal section of a Fluo4-loaded retinal slice. (B) Difference image showing Ca^{2+} changes in the INL and IPL created by subtracting the control image from the post-stimulus image (100 ms, 7 μ A). (C) Ca^{2+} changes in the amacrine cell from the INL (region 1) and IPL (region 2) plotted as a function of time.

FIGURE 6.

Simultaneous imaging of Ca^{2+} changes and elec-trophysiological recording of a synaptic current in response to epiretinal electrical stimulation in salamander retinal slice. (A) Epiretinal stimulation (100 ms, 17 μA) evoked a large, sustained synaptic current in a voltage-clamped RGC. Note the presence of transient miniature synaptic currents throughout the response. (B) Single confocal section of the retinal slice loaded with Fluo4. (C) Ca^{2+} change from a presumptive amacrine cell from the INL plotted as a function of time. The region of interest within which these Ca^{2+} changes were measured is indicated by the circle in panel B.

FIGURE 7.

Sensitivity of CoroNa Green was examined by bath application of varying concentrations of high K^+ . Panel A shows a confocal stack of a retinal slice stained with CoroNa Green (fifteen $1\ \mu m$ sections). Application of 20 mM K^+ evoked a detectable increase in fluorescence in cells throughout the retina. Panel B shows fluorescence changes plotted for a presumptive bipolar cell in the distal portion of the INL, an amacrine cell in the proximal INL, and an RGC. The corresponding regions of interest are indicated by the circles in (A). The fluorescence increase evoked by bath application of high K^+ was superimposed on a slow decrease in fluorescence due to dye bleaching.

FIGURE 8.

Simultaneous imaging of Na^+ changes and electro-physiological recording of a fast Na^+ spike evoked by epiretinal electrical stimulation in salamander retinal slice. (A) Single confocal section of the retinal slice loaded with CoroNa Green showing a portion of the IPL and GC layer. (B) Difference image subtracting a control image from the post-stimulus image reveals no detectable Na^+ changes. (C) Epiretinal stimulation (50 ms, 15 μA) evoked a fast Na^+ spike (arrow) in a voltage-clamped RGC.