Extracellular pH dynamics of retinal horizontal cells examined using electrochemical and fluorometric methods

Jason Jacoby, Matthew A. Kreitzer, Simon Alford, Haohua Qian, Boriana K. Tchernookova, Ethan Naylor, and Robert Paul Malchow

1Department of Biological Sciences, University of Illinois at Chicago, Chicago, Illinois; 2Department of Biology, Indiana University, Marion, Indiana; 3National Eye Institute, Bethesda, Maryland; and 4Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, Chicago, Illinois

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LATERAL INHIBITION is one of the most fundamental properties of neuronal processing, especially in computations that mediate visual, olfactory, auditory, and tactile sensation. In the vertebrate retina, lateral feedback inhibition mediated by horizontal cells onto photoreceptors is thought to play an important role in contrast enhancement. Despite a significant number of investigations and the passage of more than 50 years since lateral inhibition in the retina was first described, the precise molecular mechanism employed to facilitate lateral feedback inhibition remains unclear and is contentiously debated.

One hypothesis for feedback inhibition envisions that protons (H\(^+\)) released by horizontal cells lead to reduced neurotransmitter release from photoreceptors. Kleinschmidt (1991) and Barnes et al. (1991, 1993) demonstrated that synaptic feedback from photoreceptors to second-order neurons was very sensitive to changes in extracellular pH. Increases in extracellular protons were found to reduce the calcium conductance of photoreceptors, shifting the voltage of activation to more depolarized potentials. This suggests extracellular protons reduced neurotransmitter release from photoreceptors by reducing calcium entry into the photoreceptor synaptic terminal (Barnes et al. 1993). In support of this hypothesis, Hirasa and Kaneko (2003) reported that enriching the pH buffering capacity of the extracellular solution blocked the ability of horizontal cells of the newt retina to induce shifts in the calcium currents in photoreceptors. In addition, Vessey et al. (2005) reported that increased levels of the pH buffer HEPES reduced inhibitory feedback from zebrafish horizontal cells to photoreceptors as assayed by measuring calcium signals in cone synaptic terminals. Cadetti and Thoresen (2006) reported that direct hyperpolarization of horizontal cells induced a shift of the voltage dependence of the calcium conductance of photoreceptors that was abolished by increasing the pH buffering capacity with high concentrations of HEPES. Similar studies by Thoresen et al. (2008) demonstrated a similar role for H\(^+\) in feedback process onto rod photoreceptors.

The H\(^+\) hypothesis of lateral feedback inhibition predicts that depolarization of a horizontal cell should result in an acidification of the extracellular space immediately surrounding the cell due to a voltage-dependent efflux of protons. Testing this hypothesis directly by measuring pH changes within the synaptic cleft between photoreceptor and second-order interneurons has not yet proven to be possible because of the physical size (~10 nm) and complexity of this synapse (Hirasa and Kaneko 2003). In an alternate approach, researchers have focused on measuring H\(^+\) changes on the external surface of isolated retinal horizontal cells. Remarkably, conflicting results have been obtained on this topic in studies using two different techniques to measure extracellular levels of H\(^+\). Jouhou et al. (2007) used the pH-sensitive dye 5-hexadecanoylaminofluorescein (HAF) to examine changes in H\(^+\) from horizontal cells isolated from goldfish and carp. This probe has been reported to be an effective indicator of extra-
cellular pH changes in other tissues (Genz et al. 1999). Jouhou et al. showed that depolarization of horizontal cells by external potassium or glutamate altered H+ fluorescence to indicate an extracellular acidification, consistent with the H+ hypothesis of lateral feedback inhibition. However, using H+-selective microelectrodes in a self-referencing format, Molina et al. (2004) and Kreitzer et al. (2007) demonstrated that depolarization of isolated horizontal cells of skate and catfish, respectively, induced an extracellular alkalization, precisely opposite to the result predicted by the H+ hypothesis.

The discrepancy between these findings could be due to differences in the methods used (fluorescent imaging vs. self-referencing), the species used (carp/goldfish vs. catfish/skate), the extracellular buffer used (bicarbonate vs. HEPES), and whether the experiments were performed in the presence (Jouhou et al. 2007) or absence (Kreitzer et al. 2007; Molina et al. 2004) of cobalt in the Ringer solutions. To resolve these issues, in the present work we have used both techniques to examine alterations in H+ flux from horizontal cells of a single species in identical experimental conditions. Both self-referencing and fluorescent imaging techniques were applied to cone-driven horizontal cells from catfish (*Ictalurus punctatus*) that were identified by their morphological characteristics (Kaneko and Stuart 1984). To control for experimental differences, both imaging and self-referencing techniques were performed using 1 mM HEPES in the absence of cobalt. We report here that self-referencing H+-selective microelectrodes still report an extracellular alkalization under these conditions, whereas ratiometric imaging studies using HAF report an acidification. We present evidence suggesting that the change in pH reported by HAF is an intracellular acidification. We interpret our data as most consistent with the activation of a plasmalemma calcium pump (PMCA), which acidifies the cytoplasm of the cell while promoting an extracellular alkalization. Our results thus argue against the H+ hypothesis of lateral feedback inhibition in the outer retina.

**MATERIALS AND METHODS**

*Cell dissociation.* The retinal dissociation protocol used in this study was similar to that reported in Kreitzer et al. (2007). All experiments were conducted following animal care protocols approved by the University of Illinois at Chicago, the Marine Biological Laboratory, and Indiana Wesleyan University. Channel catfish (*I. punctatus*) between 6 and 10 in. were obtained from Osage Catfisheries (Osage Beach, MO), Jones Fish Hatchery (Newtown, OH), or Keystone Fish Hatcheries (Richmond, IL). Fish were kept at room temperature for up to 2 mo and were dark-adapted for at least 1 h before dissection. Catfish were anesthetized in 3.8 liters of water supplemented with 1 g of tricaine methanesulfonate (MS-222; Argent) and 1.5 g of sodium bicarbonate (S233; Fisher) for 5–10 min. The spinal cord was cervically transected and the brain pithed. Eyes were enucleated and cut in half, and the posterior portion containing the retina was immersed in catfish Ringer solution consisting of (in mM) 126 NaCl, 4 KCl, 3 CaCl2, 1 MgCl2, 10 HEPES, and 15 glucose. Whole retinas were removed and agitated in a dissociation solution containing 1.2 mg/ml papain (LS003119; Worthington) and 0.5 mg/ml cysteine (C7755; Sigma) for 25–30 min. After eight rinses with catfish Ringer solution, retinas were mechanically triturated with a graduated 5-ml pipette, generating a retinal cell suspension; 1–2 drops of this suspension were placed in 35-mm culture dishes (Falcon 3001) preloaded with 3 ml of catfish Ringer solution. Recordings were made from cells whose nearest neighbors were at least 1 mm distant. Dishes were maintained at 14°C for up to 2 days. All experiments on isolated cells were conducted at room temperature (~18–21°C).

*Preparation of H+-selective electrodes.* H+-selective microelectrodes were prepared as described in Kreitzer et al. (2007) (see Smith et al. 1999 for a more complete description). Silanized pipettes with tip diameters of 2–4 μm were back-filled with a solution composed of 100 mM KCl and 10 mM HEPES, adjusted to a pH of 7.0 with KOH. The microelectrode tip was front-filled (~30-μm column) with a highly H+-selective resin (hydrogen ionophore 1-cocktail B; Fluka Chemical). This resin is reported to be greater than 105 times more sensitive to H+ than to Na+ or K+ (Fluka 1996). As reported in previous studies, both the extracellular voltage fields and membrane surface charges associated with natural cellular currents would be unlikely to contribute to the signals we report in the self-referencing format, due to the small size of these naturally occurring voltages (nanovolt range) and the distance of the H+-selective electrode from the cell membrane (1–2 μm). As noted in Molina et al. (2004), the measured H+ gradients detected from isolated horizontal cells extend tens of micrometers away from the cell.

*Self-referencing recordings.* H+-selective microelectrodes were used in a self-referencing format (Smith and Trimarchi 2001; Smith et al. 2007). This allows for a differential measurement of proton concentration by moving the electrode between a position adjacent to the cell membrane where a voltage reading is taken and a set distance away where a second reading is taken. This differential signal reflects the difference in H+ concentration at the two locations. This procedure results in the elimination of much of the slow electrical drift inherent in the signal from such ion-selective electrodes, since the drift that is common to the two positions measured is subtracted out. An important assumption underlying this method is that the movement of the electrode is fast relative to the rate of electrical drift, but not fast enough to mechanically disturb the diffusional gradient of H+ ions. When these conditions are met, self-referencing effectively filters out electrical interference caused by random slow electrical drift. This procedure, combined with extensive averaging of the signal, has been estimated to increase the useful sensitivity of these electrodes by as much as 1,000 times (Somieski and Nagel 2001). In these experiments, microelectrodes were moved alternately between a point close (within 1–2 μm) to the cell membrane and a known distance away (typically 30 μm). The frequency of movement was 0.3 Hz, a value chosen to obtain measurements as rapidly as possible while minimally disturbing the solution by stirring and mixing, which would diminish or abolish the proton gradient we are attempting to measure (cf. Kuhthreiber and Jaffe 1990). Thus the time resolution of these measurements is on the order of seconds, similar to the acquisition of fluorescent imaging signals using the pH-sensitive dye HAF as described below, and for both methods employed, changes on the millisecond time scale will not readily be detected. Electronics, software, and mechanical control of electrode movement were the same as described in Molina et al. (2004) and were products of the BioCurrents Research Center (Marine Biological Laboratory, Woods Hole, MA). Electrodes were calibrated using commercially purchased pH standards: pH 6.00, 7.00, and 8.00 (SB104, SB108, and SB112, respectively; Fisher Scientific). Only electrodes possessing Nernst slopes between 45 and 60 mV/pH unit were used.

*Self-referencing experimental protocol.* The experimental protocol used here is identical to that implemented in Kreitzer et al. (2007). Measurement of proton fluxes from isolated cells relies on the establishment of a proton gradient generated at the outer membrane that declines by diffusion away from the cell. The small extracellular H+ gradients expected to be generated by isolated cells would likely be disturbed or eliminated by rapid superfusion of solutions around the cell. Consequently, we applied solutions with a handheld pipette and then allowed the solution to settle. The extracellular solution used in most experiments consisted of (in mM) 126 NaCl, 4 KCl, 3 CaCl2, 1 MgCl2, 1 HEPES, and 15 glucose; pH was adjusted to 7.60 with NaOH (all chemicals were purchased from Sigma-Aldrich unless otherwise stated).
otherwise indicated). A H⁺-selective electrode was placed ~2 µm from the cell membrane of a cone horizontal cell identified by its characteristic morphology (Dong et al. 1994). Differential extracellular recordings were made for several minutes to ensure a steady baseline reading. Normal extracellular solution (1 mM) was then applied to ensure that application of the fluid itself did not alter the measured proton flux. The application of the solution required entrance into the Faraday cage, which resulted in large artifactual transients during solution application; these portions of the traces were subtracted and are indicated as discontinuities in the traces presented. Several minutes later, the same solution containing the test compound was added. The pH of the solution containing the test compound was adjusted to match the normal extracellular solution to within 0.01 pH units. All solutions used in the experiments presented here were maintained at a pH of 7.6. The concentrations listed throughout reflect the final concentration of the drug after complete mixing unless otherwise noted. All drugs typically remained in the dish during the remainder of the recording except where noted.

Two separate control experiments were conducted to ensure that drugs did not alter the ability of H⁺-selective electrodes to sense changes in pH. First, proton gradients were measured from H⁺ source pipettes (Molina et al. 2003) in the presence and absence of a drug. The second type of control experiment involved calibration of electrodes at pH 6.0, 7.0, and 8.0 in the absence and presence of the drug.

Ratiometric epifluorescent imaging protocol. HAF (MW 585.7; Invitrogen Molecular Probes, Eugene, OR), a pH-sensitive fluorescent dye, was used to monitor changes in pH using fluorescent imaging techniques (Genz et al. 1999). The lipophilic hydrocarbon tail of the HAF molecule is thought to imbed itself into the external side of the plasma membrane and project the pH-sensitive fluorescein moiety ~2 nm outward from the cell surface to report changes in extracellular pH (Jouhou et al. 2007). The HAF staining protocol used in these experiments was identical to the protocol described in Jouhou et al. (2007). A stock solution was created by dissolving 1 mg of HAF in 500 µl of dimethyl sulfoxide (DMSO), and aliquots were placed into the freezer. HAF stock solutions were diluted in catfish Ringer solution to 5 µM, and isolated horizontal cells were incubated in HAF-containing Ringer solution in the dark for 20 min at 4°C. Cells were imaged immediately following full wash off with dye-free Ringer solution. Epifluorescent images were collected from a charge-coupled device camera (Hamamatsu ORCA) mounted onto a Zeiss LSM-510 NLO META, equipped with an inverted LUMPlanFL 40/1.1, both present at the Marine Biological Laboratory. In experiments using the Zeiss LSM-710 microscope, cells were plated on Ibidi 81156 culture dishes with thin plastic bottoms (~180 µm thick) and illuminated from below. Images were acquired using Zeiss Zen LE 2009 and 2010 software and analyzed further using ImageJ. The HAF staining protocol was again taken from the methods of Jouhou et al. (2007). For obtaining experiments employing both HAF and the dye MitoTracker Deep Red 633, cells were stained with HAF as described above and following wash off were subsequently stained with MitoTracker. A stock solution of MitoTracker was created by dissolving 50 µg of MitoTracker into DMSO (concentration of 1 mM). A final working concentration diluted to 100 nM was added to the cell culture dish and incubated in the dark at room temperature for 20 min. Costained cells were imaged immediately following MitoTracker wash off with dye-free Ringer solution; HAF fluorescence was measured at 488 nm (emission collected at 530 nm and above) followed by MitoTracker stained at 633 nm (emission collected at 665 nm and above).

In experiments examining staining of the dye FM 1-43, a 5 mM stock solution was prepared in distilled water. Stock solution (2 µl) was added directly to the cell culture dish containing 2 ml of Ringer solution, leading to a final working concentration of 5 µM. Cells were plated on Ibidi 81156 culture dishes and imaged 5–10 min after being loaded on a Zeiss LSM-710 NLO confocal microscope. A single excitation wavelength was used (458-nm argon) through an inverted C-Apochromat ×40/0.1 water-immersion lens. Emission wavelengths above 510 nm were collected, and images were obtained using Zeiss Zen 2010 acquisition software.

Confocal ratiometric imaging using HAF was collected using either a Leica SP2 with a 10 objective or a Zeiss LSM-710 NLO equipped with an inverted 40 water-immersion objective (C-Apochromat ×40/0.1). Images were acquired using Leica LCS or Zeiss Zen software and analyzed using ImageJ. Argon laser lines excited the dye alternately between 458- and 488-nm excitation wavelengths at 5-s intervals. Both emission spectra were collected at 530 nm and above, and the fluorescence ratio was plotted. Glutamate-containing Ringer solution was hand-pipetted into the cell culture dish in the same fashion used in the self-referencing technique so as not to disturb the local proton gradient present on the cell surface. Raw data obtained through confocal ratiometric imaging were overlaid by data smoothed by a four-nearest-neighbor average.

Data treatment and statistical analysis. Student’s paired t-tests were used throughout to determine statistical significance, with a criterion of P < 0.01 selected as indicating significantly different distributions. Data are means ± SE throughout. For most experiments, values reported reflect the average of the 10 points before application of a drug and the 10 points after application. All data were statistically analyzed and graphically displayed using Prism 5 (GraphPad Software).

RESULTS

Calibrations of two techniques. Calibrations were performed with both pH-selective electrodes and the pH-sensitive dye HAF to establish their relative sensitivity to changes in proton concentration. H⁺-selective microelectrodes were calibrated by immersing the electrode tip in solutions of different known pH values. A value change of approximately ~58 mV was observed when the pH was increased by 1 log unit (Fig. 1A). This result was consistent with the expected linear Nernst slope for a functioning proton-selective microelectrode. Only electrodes with Nernst slopes between 45 and 60 mV/pH unit were used. For the fluorescent imaging technique, the pH-sensitive dye HAF was calibrated by measuring the relative fluorescence of HAF-stained horizontal cells bathed in solutions of
known pH values. Cone-driven horizontal cells were treated with gramicidin to eliminate biological activity and stained with HAF, following the same protocol as performed by Jouhou et al. (2007). Relative fluorescence from a circular portion of the central soma with a diameter of \(20\, \mu\text{m}\) was selected for analysis in response to 488-nm/440-nm excitation. HAF calibration performed on a gramicidin-treated cell reports a decrease in the fluorescence ratio as the pH of the extracellular solution is acidified (Fig. 1B), similar in magnitude to the values observed by Jouhou et al. (2007). A graph plotting the average calibration for HAF from four cells is shown in Fig. 1C.

Self-referencing technique reports an extracellular alkalinization with cells bathed in high extracellular potassium. Experiments were performed using self-referencing pH-selective electrodes to examine alterations in extracellular proton concentration after isolated cone horizontal cells of catfish were exposed to high extracellular potassium. Self-referencing recordings of H\(^+\) flux from these cells were made by subtracting the signals from two points, one 1–2 \(\mu\text{m}\) away from the cell and another 30 \(\mu\text{m}\) distant (see Fig. 2A). This produced a differential signal reflecting the difference in proton concentration at the two locations (Kreitzer et al. 2007; Molina et al. 2004). A standing positive differential signal, in this case around 50

![Fig. 1](image1.png)

**Fig. 1.** Calibration curves for self-referencing electrodes and changes in 5-hexadecanoylaminofluorescein (HAF) fluorescence. A: calibration of a proton-selective microelectrode bathed in solutions of different pH values. An approximate 58-mV difference was observed between each pH unit. B: pH-sensitive dye HAF calibration via fluorescent imaging. Cells were treated with gramicidin, stained with HAF, and serially perfused with extracellular Ringer solutions of known pH values. Cells were alternately stimulated with 488- or 440-nm light; fluorescence emitted at 530 nm and beyond was collected. A circular area of \(20\, \mu\text{m}\) in the central portion of the soma was selected as the region of interest, and the ratio of light emitted at 488 nm/440 nm was plotted. C: average calibration values for 4 cells treated with HAF.

![Fig. 2](image2.png)

**Fig. 2.** Self-referencing and fluorescent imaging consistently report opposite results. A: photomicrograph of a horizontal cell from the retina of a catfish with an H\(^+\)-selective microelectrode placed several micrometers away from the cell surface. Scale bar, 60 \(\mu\text{m}\). B: self-referencing consistently reported an alkalinization of the space immediately adjacent to the cell membrane following application of high extracellular potassium (45 mM final concentration). Background control is marked by the asterisk, where the microelectrode was moved 200 \(\mu\text{m}\) distant from the cell. C: an isolated horizontal cell stained with pH-sensitive dye HAF (pseudocolor). Scale bar, 25 \(\mu\text{m}\). D: fluorescent imaging using HAF consistently reported an acidification response to superfusion of 100 mM K\(^+\)-containing Ringer solution. Decrease in fluorescence ratio is associated with an acidification.
μV in magnitude, was consistently detected from cells before stimulation (Fig. 2B). These positive values indicate that the extracellular solution adjacent to the cell membrane has a higher concentration of protons than the location 30 μm distant. Bath application of a Ringer solution in which 45 mM KCl replaced 45 mM NaCl led to an alteration in the differential voltage of the pH-selective electrode. The addition of high external potassium consistently led to a marked drop in the self-referencing signal to a negative voltage difference, indicating that the solution adjacent to the cell membrane was now more alkaline than the solution some 30 μm away (Kreitzer et al. 2007). In 5 cells, the standing proton flux changed from 80 ± 15 to −57 ± 23 μV immediately following the addition of high-potassium Ringer solution. After 30 s with high potassium still in the bath, the differential signal averaged −33 ± 18 μV. The values at both time points following addition of high potassium are significantly different from the value of the standing proton flux before depolarization. A background control was performed in each experiment where the microelectrode was moved 200 μm from the cell surface (asterisk in Fig. 2B); at this location, the solution pH should be the same at the two electrode positions and the differential signal should be close to zero. This was indeed the case, with the background control averaging 0.256 ± 0.663 μV.

We have previously presented evidence that the standing proton flux of nonstimulated cells reflects in large part the activity of a Na+/H+ exchanger, since the proton flux is reduced by EIPA, an analog of amiloride that blocks Na+/H+ exchangers, and is also reduced by replacement of all of the sodium in the bath with the larger cation, NMDG (Kreitzer et al. 2007; Molina et al. 2004). This presents a potential problem in the interpretation of the above results obtained following the potassium stimulation protocol employed by Jouhou et al. (2007). In this protocol, sodium in the Ringer solution was replaced by potassium. Removing extracellular sodium has the potential to reduce Na+/H+ exchange activity, which would be seen as a reduced extracellular acidification adjacent to the cell membrane. To eliminate this potential problem, we also conducted experiments in which cells were bathed in a Ringer solution in which 41 mM sodium had been replaced with 41 mM choline to eliminate potential alterations in Na+/H+ exchange. To stimulate the cells, the choline solution was replaced with one containing an equimolar amount of potassium, resulting in a total concentration of 45 mM extracellular potassium and leaving extracellular sodium levels unchanged. We again observed a change in fluorescence in the HAF signal indicative of an acidification; in 5 cells, there was an average normalized reduction in fluorescence of −0.18 ± 0.02 ΔF/F before wash off (normalized to the prestimulus fluorescence).

Extracellular glutamate mimics high extracellular potassium-induced effects. Self-referencing measurements demonstrated that addition of extracellular glutamate, the neurotransmitter believed to mediate excitatory synaptic transmission between photoreceptor and horizontal cells, induced an extracellular alkalinization of isolated cone horizontal cells similar to that induced by high extracellular potassium. Figure 3A shows that when glutamate was added to the extracellular bath solution at a final concentration of 100 μM, a significant change in the differential signal measured by the H+-selective microelectrode was detected, indicating an alkalinization next to the cell’s surface. In 6 cells, the mean standing signal was 118 ± 24 μV. Immediately following the addition of glutamate, the peak amplitude of the differential voltage signal was −56 ± 17 μV. After 30 s of glutamate in the bath solution, the differential signal averaged −13 ± 12 μV. The results presented here (Figs. 2B and 3A) using the self-referencing technique are consistent with the findings published by Molina et al. (2004) and Kreitzer et al. (2007), which demonstrated extracellular alkalinizations from skate and catfish horizontal cells, respectively, when challenged with glutamate. Although an exhaustive analysis of electrode position on pH responses was not done, we did not observe any consistent differences dependent on the orientation of placement of the electrode near the cell in the extracellular alkalinization responses induced by either glutamate or high extracellular potassium.

Epifluorescent imaging of HAF indicates an acidification in response to high extracellular potassium. Cone-driven horizontal cells from catfish retina were isolated and stained with the fluorophore HAF using the identical protocol employed by Jouhou et al. (2007) (Fig. 2C). Ringer solution was superfused continuously over the cell. Following the stimulation protocol of Jouhou et al. (2007), cells were challenged with a 100 mM K+-containing Ringer solution. This high extracellular potassium resulted in a marked decrease in the ratio of 488-nm/440-nm emitted fluorescence, indicative of an acidification (Fig. 2D; refer to calibration in Fig. 1B). This result was very similar to that reported by Jouhou et al. (2007). Six HAF-stained cells were analyzed and had an average normalized reduction in fluorescence of −0.11 ± 0.02 ΔF/F before wash off (normalized to the prestimulus fluorescence). With the use of the HAF calibration curve (Fig. 1B), the pH change was estimated to be −0.16 ± 0.03 pH unit for cells stimulated with 100 mM K+.
In contrast, measurement of glutamate-induced changes in HAF fluorescence revealed that extracellular glutamate produced a reduction in the fluorescence ratio indicative of an acidification, similar to what was observed with high extracellular potassium. This result was consistent in 5 cells challenged with 100 μM glutamate. The reduction in fluorescence ratio averaged $-0.08 \pm 0.02 \Delta F/F$ at its most acidic point before wash off (normalized to prestimulus level of fluorescence). The pH changes reported by HAF are estimated to be $-0.12 \pm 0.02$ pH units for all cells stimulated with 100 μM glutamate compared with the HAF calibration. These findings are also remarkably similar to those obtained by Jouhou et al. (2007) on stimulation of goldfish and carp horizontal cells with glutamate. No statistically significant difference was found between the magnitude of the glutamate- and high potassium-induced reductions in HAF fluorescence.

**HAF staining does not negate the extracellular alkalinization reported by self-referencing.** Self-referencing electrodes were used to measure extracellular pH from cone-driven horizontal cells that had been stained with HAF. This experiment was performed to examine the potential interference of HAF on mechanisms necessary for inducing extracellular alkalinization. Despite being stained with HAF in the same fashion as done for the imaging studies, self-referencing electrodes continued to report a robust extracellular alkalinization in the presence of glutamate (100 μM, Fig. 3B). This suggests that the cellular machinery responsible for facilitating an extracellular alkalinization response to depolarization remains intact even after HAF loading. The mean standing signal from pH-selective electrodes for cells stained with HAF was $104 \pm 17 \mu V$, and the peak amplitude of the differential voltage signal changed to $-55 \pm 26 \mu V$ immediately following the addition of 100 μM glutamate. After 30 s in the presence of glutamate, the differential signal was $-6 \pm 20 \mu V$. No significant difference was found between HAF stained and unstained cells for the standing signal, peak amplitude after glutamate, or 30 s after the application of glutamate (Fig. 3C).

Confocal microscopy reveals HAF staining within the intracellular compartment of the cells. Our results thus far show that the two different techniques, self-referencing pH-selective electrodes and fluorescence changes from the pH-sensitive dye HAF, report opposite results when cells are stimulated by glutamate or high extracellular potassium; self-referencing pH-selective electrodes report an extracellular alkalinization, whereas changes in HAF fluorescence indicate an acidification. One hypothesis that could explain these results is that HAF is not reporting exclusively extracellular pH alterations, but might also be responding to changes in intracellular pH. This could occur if the dye was not located exclusively to the extracellular face of the plasma membrane of the cell but was present in the cell interior as well. To determine whether HAF was isolated to the exterior surface of the cell membrane in retinal horizontal cells, we stained cells with HAF per the protocol used by Jouhou et al. (2007) and imaged cells using laser scanning confocal microscopy. If HAF was solely embedded in the plasma membrane, we would expect to see a fluorescent outline of the cell in an optical section obtained from the center of a z-stack made through the cell. Figure 4A shows a high-magnification image slice derived from a central section of a cell’s z-stack profile revealing instead extensive staining throughout the cell’s interior. A lower magnification image of a center slice of a second HAF-stained cell is shown in Fig. 4B, and again shows extensive staining in the interior of the cell. To the top and right of the stained cell in Fig. 4B is the series of image stacks obtained in the z-plane along the x- and y-axes, which again makes clear the extensive amount of intracellular staining of HAF that is present. To confirm that our instrumentation and technique were capable of detecting the presence of a dye localized primarily to the external face of the horizontal cells, we examined the pattern of fluorescence of cells stained with the lipophilic dye FM 1-43. The center image of Fig. 4C shows the expected clear pattern of ring-shaped staining along the edge of the cell, indicating that the dye is indeed localized primarily to the external face of the membrane. The z-stack of images above and to the right of the cell
in Fig. 4C further emphasize the extracellular localization of FM 1-43.

To further verify that HAF was indeed localized within the intracellular space of the horizontal cells, we costained cells with HAF and a mitochondrion-selective probe, MitoTracker Deep Red 633. MitoTracker was excited at 633 nm, and emission from the dye was measured at 665 nm, creating a spectrum gap from HAF excitation and emission and minimizing potential cross-over signals during costaining trials. Examination of the channel exhibiting HAF fluorescence in a costained cell again revealed HAF infiltration and extensive staining within the cell’s interior in a thin optical slice (shown in green, Fig. 5A). Figure 5B illustrates the staining pattern for MitoTracker in the same cell and shows extensive staining of organelles within the intracellular space. The merged image of HAF (green) and MitoTracker (red) demonstrates a high degree of colocalization of these dyes (yellow) as depicted in Fig. 5C. The staining of HAF throughout the remainder of the cell can still be observed in green. The costained images obtained using confocal microscopy strongly suggest that HAF is not isolated to the immediate extracellular surface of the cell membrane, but rather is found at a high concentration inside the cell.

**HAF reports an intracellular acidification.** The presence of HAF staining within the intracellular compartment suggests that epifluorescence measurements may be reporting pH changes from the cell’s interior. To test this hypothesis, we used confocal microscopy to monitor changes in fluores-

Fig. 4. Confocal microscopy reveals HAF staining within the intracellular compartment. A: image taken from an optical section approximately in the center of a stained horizontal retinal cell in the $z$-axis. Scale bar, 20 μm. B: orthographic projection derived from a $z$-stack of a second HAF-stained cell. Center slice showing extensive intracellular staining in a second cell is flanked at top and at right by orthographic projections showing extensive intracellular staining throughout the cell interior. Scale bar, 20 μm. C: cell stained with FM 1-43; note extensive ring of extracellular staining in the center optical slice and the lack of significant levels of intracellular staining in the orthographic projections derived from a $z$-stack of the cell to the top and right. Scale bar, 20 μm.

Fig. 5. Costaining with HAF and the dye MitoTracker Deep Red confirms staining of HAF within intracellular compartment. Confocal images were taken approximately in the center of the cell within the $z$-plane. A: fluorescence of HAF at 488 nm. B: staining pattern of MitoTracker Deep Red at 633 nm. C: merged image of HAF and MitoTracker; areas of dye colocalization appear yellow. Scale bar, 20 μm.
Block of the extracellular alkalinization by nifedipine and carboxyeosin. Our previous studies (Kreitzer et al. 2007; Molina et al. 2004) presented evidence that the extracellular alkalinization detected by self-referencing $H^+$-selective electrodes on stimulation with high extracellular potassium or glutamate was calcium dependent, and this was interpreted to indicate activation of a plasmalemma $Ca^{2+}/H^+$ ATP-driven pump. Experiments in support of this hypothesis included the ability of low extracellular calcium to reduce the extracellular alkalinization induced by high potassium and the ability of 10 $\mu$M of the calcium channel blocker nifedipine to prevent the alkalinization normally induced by high potassium or glutamate from occurring. In the present set of experiments, we extended these observations by examining the ability of the calcium channel blocker nifedipine, the PMCA blocker carboxyeosin, and lanthanum to block the extracellular alkalinization once it had been initiated by high extracellular potassium. Figure 7A shows the response from one cell on stimulation by high potassium by the exchange of 41 mM extracellular NMDG with 41 mM potassium. Before the application of high potassium, the typical standing acidic proton flux was observed, and when the solution was switched to high potassium, the now familiar alkalinization followed. On addition of 30 $\mu$M nifedipine, the voltage reported by the self-referencing electrode jumped positive, much closer to the value for the initial standing acidic proton flux. A similar ability of nifedipine to alter the signal reported by the self-referencing electrode was seen in seven other cells, and the average results are plotted in Fig. 7B, showing that 30 $\mu$M nifedipine was able to completely abolish the alkalinization that had been induced by high extracellular potassium.

Prior data (Kreitzer et al. 2007) supporting a role for the PMCA in mediating the extracellular alkalinization of catfish horizontal cells relied on the use of carboxyeosin, a compound known to act as a PMCA antagonist (Choi and Eisner 1999; Fierro et al. 1998; Wanaverbecq et al. 2003). In the prior study, cells were first preincubated in carboxyeosin for several minutes before the application of glutamate; following this incubation from an optical section derived from the center of a $z$-stack. Measurements of intracellular changes in HAF fluorescent intensity following the application of glutamate mimicked the acidification response to glutamate in epifluorescent trials. The representative trace presented in Fig. 6 shows that on addition of glutamate (final concentration of 100 $\mu$M), this HAF-stained cell reported a decrease in the fluorescence ratio of intracellular HAF. Eight HAF-stained cells were analyzed and had an average normalized reduction in fluorescence ratio of $-0.10 \pm 0.02$ $\Delta F/F$. Cellular debris outside of the horizontal cell but within the same focal plane showed HAF staining but no alteration in fluorescence in the presence of glutamate. Every cell used in these experiments yielded substantial HAF staining and infiltration within the intracellular space throughout the $z$-axis. These results strongly suggest that HAF is reporting an intracellular acidification in response to the application of glutamate.

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bation period, glutamate addition no longer induced an extracellular alkalinization. We extended this observation in the present set of experiments by first adding glutamate to initiate the extracellular alkalinization and then looking to see if later application of carboxyeosin would reduce the glutamate-induced alkalinization. Figure 7C shows an example from one cell obtained using this experimental paradigm; indeed, addition of 10 µM carboxyeosin diacetate abolished the alkalinization induced by glutamate. Figure 7D presents results from 11 cells using the same experimental paradigm. In cells not treated with carboxyeosin and observed over similar lengths of time, the self-referencing signal remained alkaline.

Lanthanum, another compound reported to be able to block plasmalemma calcium ATPase activity (Hersher and Rega 1988), also abolished the glutamate-induced alkalinizations from isolated catfish horizontal cells. Cells bathed in 1 mM lanthanum for ~5 min had an average standing proton signal of 139.2 ± 14.44 µV (n = 5); the differential signal following application of 100 µM glutamate was 146.6 ± 14.24 µV (n = 5), a value not significantly different from that of the initial, preglutamate condition. Although it is well known that lanthanum can block a number of different channels (including calcium channels) in addition to blocking PMCA activity (cf. Alshuaib and Matthew 2005; Nathan et al. 1988), its ability to abolish the alkalinization induced by glutamate is consistent with the hypothesis that the proton fluxes we have observed result from the activation of a plasmalemma calcium pump.

Reduction of the alteration in HAF fluorescence by low extracellular calcium. Our results thus far suggest that the alteration of fluorescence reported by HAF on stimulation of cells by high extracellular potassium or glutamate likely reflects an internal acidification. This would be expected on stimulation of a plasmalemma Ca2+/H+ antiporter ATP-driven pump, activation of which should produce an extracellular alkalinization and an intracellular acidification. We have previously published evidence that the self-referencing alkalinization induced by glutamate and potassium is significantly diminished if extracellular calcium is removed from the bath (Kreitzer et al. 2007; Molina et al. 2004). In the present work, we also observed that the alteration in HAF fluorescence was significantly diminished in solutions in which the normal 3 mM extracellular calcium was replaced by 3 mM magnesium (nominally 0 mM calcium). In these experiments, cells were stimulated by high potassium by exchanging 41 mM extracellular choline with 41 mM potassium (note that no calcium chelators were applied to the bath; thus there is likely a small residual of calcium in the nominally 0 mM calcium solution). The dark line in Fig. 8A shows the change in fluorescence in a cell bathed in normal calcium, whereas the lighter line shows the reduced change in fluorescence observed in a cell bathed in the nominally 0 mM extracellular calcium solution. Figure 8B shows the quantitative difference observed in cells bathed in normal and nominally 0 mM extracellular calcium. In 5 cells with the normal amount of 3 mM extracellular calcium present, an average normalized reduction in fluorescence of −0.18 ± 0.02 ΔF/F was observed. In 5 other cells bathed in a solution in which 3 mM magnesium had replaced the normal 3 mM calcium, the alteration in HAF fluorescence induced by 45 mM potassium was reduced to only −0.03 ± 0.01 ΔF/F.

DISCUSSION

Self-referencing H+-selective microelectrodes and the pH-sensitive dye HAF reported different changes in H+ from isolated retinal horizontal cells. H+-selective electrodes consistently reported an extracellular alkalinization when cells were challenged by high extracellular potassium or glutamate, consistent with previous studies on skate and catfish retinal horizontal cells (Kreitzer et al. 2007; Molina et al. 2004). Alterations in fluorescence of the pH-sensitive dye HAF induced by these same treatments invariably reported an acidification, similar to the data reported by Jouhou et al. (2007). The difference in the direction of the reported pH change cannot be explained by differences in species, recording conditions, or the type of extracellular pH buffer employed, since in the present set of experiments both techniques examined changes from isolated cone-driven horizontal cells of catfish under similar experimental conditions. Examination of the cellular distribution of HAF using confocal microscopy revealed that this pH-sensitive dye was not exclusively localized to the extracellular side of the plasma membrane. Rather, extensive staining was seen throughout the interior of the cell. Measurements of fluorescence changes in optical sections restricted to dye in the interior of the cells also reported an acidification. Finally, removing extracellular calcium diminishes both the self-referencing alkalinizations (Kreitzer et al. 2007) and the acidifications reported by alterations in HAF fluorescence. These observations suggest that the fluorescence alterations observed with HAF in cells challenged with high external potassium or glutamate likely reflect changes in intracellular pH rather than extracellular alterations in H+.

Fluorescein and related dyes based on this compound, such as eosin, have been reported to block the activity of plasmalemma calcium pumps (Gatto and Milanick 1993; Gatto et al. 1995). As a variant of fluorescein, HAF itself could have
reduced PMCA activity, thus decreasing the transport of protons from the outside of the cell to the cell interior. However, as shown in Fig. 3, self-referencing recordings from cells that are stained with HAF still report an extracellular alkalinization when challenged with either high potassium or glutamate. We conclude that the cellular machinery responsible for inducing the extracellular alkalinization process remains intact even after HAF staining, and we hypothesize that the presence of HAF does not significantly alter PMCA function in retinal horizontal cells. It is worth noting that eosin is a reversible inhibitor of the PMCA pump and that the experiments by Gatto et al. (1993, 1995) were done by bathing vesicles continuously with eosin in the bath. In the experiments employing HAF, the dye was applied to the cell and then rinsed out of the dish so that cells were not continuously exposed to extracellularly dissolved HAF in the bath during the imaging experiments.

An intracellular acidification would be expected from the model proposed by Molina et al. (2004) and Kreitzer et al. (2007) to explain the extracellular alkalinization observed with self-referencing pH-selective microelectrodes on depolarization of cells by high external potassium and glutamate. In this model, depolarization of isolated horizontal cells by high potassium leads to the opening of voltage-gated L-type calcium channels and entry of significant amounts of calcium into the cell. This in turn results in an increase in the activity of plasmalemma calcium Ca\(^{2+}/H^+\) ATPase (PMCA) pumps, which expel intracellular calcium and take up protons from the extracellular space. This would make the interior of the cell acidic (Hao et al. 1994; Salvador et al. 1998). Extracellular glutamate would also increase intracellular calcium by depolarizing the cell and activating L-type calcium channels, but it would also allow calcium entry directly through calcium-permeable AMPA receptors and NMDA receptors known to be present in catfish cone horizontal cells (Linn and Christensen 1992; O’Dell and Christensen 1986). This increased calcium load would also likely promote the activity of PMCA pumps, which would be expected to make the extracellular solution adjacent to the cell membrane alkaline while inducing an intracellular acidification. Studies using the intracellular pH indicator dye BCECF have already shown that the interior of catfish and skate retinal horizontal cells becomes more acidic when cells were challenged with extracellular glutamate (Dixon et al. 1993; Molina et al. 2004). In the present work, we also demonstrated a decrease in the fluorescence ratio of HAF from the center optical section of horizontal cells examined using confocal microscopy, indicative of an intracellular acidification (Fig. 6). The internal acidification in retinal horizontal cells induced by activation of glutamate receptors on horizontal cells was significantly reduced when extracellular calcium was removed from the bath (Molina et al. 2004).

The ability of the PMCA blocker 5(6)-carboxyeosin to eliminate the extracellular alkalinization induced by glutamate (Kreitzer et al. 2007 and the present work, Fig. 7, C and D) lend additional support for the hypothesis that the extracellular H\(^+\) alkalinization detected by self-referencing H\(^+\)-selective electrodes reflects an increase in PMCA activity. Furthermore, the ability of high potassium, glutamate, and direct depolarization to induce an extracellular alkalinization is blocked when calcium is removed from the extracellular solution or when nifedipine, an inhibitor of L-type voltage-gated calcium channels, is present in the bath solution (Kreitzer et al. 2007; Molina et al. 2004; and the present work, Fig. 7, A and B). Molina et al. (2004) also demonstrated that increasing intracellular calcium, by photolysis of the caged calcium compound NP-EGTA, also induced an extracellular alkalinization. Taken together, the data argue that the extracellular alkalinization is critically dependent on an increase in intracellular calcium and that the alkalinization results from the removal of calcium from the cell by activation of the PMCA Ca\(^{2+}/H^+\) pump. Recent experiments in hippocampal slices measuring surface alkaline transients from voltage-clamped CA1 pyramidal cells demonstrated that the rapid alkalinization evoked by synchronous activation also appears to be mediated by the activity of a plasma membrane calcium ATPase (Makani and Chesler 2010).

Based on the ability of the V-type ATPase inhibitor bafilomycin to block the changes in HAF fluorescence in isolated retinal horizontal cells, Jouhou et al. (2007) argued that a depolarization-induced activation of a vacuolar ATPase led to the acidifications they reported using HAF. However, this interpretation rests on the assumption that the dye HAF is reporting extracellular acidification and that bafilomycin is acting on an ATPase present in the external face of the membrane. As we have shown, HAF is likely to be reporting an intracellular acidification. Moreover, the addition of bafilomycin is likely to have significant effects on intracellular V-ATPases in a number of intracellular compartments, leading to potentially significant alterations in intracellular levels of pH and widespread effects on a number of cellular parameters (Zhdanov et al. 2011). Thus the effects of bafilomycin are unlikely to be clear indicators of the functioning of an extracellularly facing V-ATPase.

Our results help to resolve the puzzling discrepancy between the findings from horizontal cells obtained with the pH-sensitive dye HAF and pH-selective microelectrodes, and suggest that depolarizing agents induce an extracellular alkalinization and an intracellular acidification in isolated horizontal cells. These findings argue strongly against the hypothesis that H\(^+\) release from horizontal cells mediates lateral inhibition in the outer retina. Despite this statement, we believe that further studies in the intact retina are essential to determine how these findings fit into models of pH regulation of synaptic transmission in the intact retina. It is conceivable that microdomains of extracellular pH regulation exist within the retina and that such microdomains might be present within the synaptic pedicles and spherules of individual photoreceptors. Our self-referencing electrodes cannot be used to study microdomains that might exist in the submicron spacing in the complex neuropil where photoreceptor, horizontal cell, and bipolar cell processes intertwine and interact. Indeed, a number of studies have presented strong evidence in support of the H\(^+\) hypothesis for lateral inhibition (Cadetti and Thoreson 2006; Crook et al. 2011; Davenport et al. 2008; Hirasawa and Kaneko 2003; Thoreson et al. 2008; Vessey et al. 2005). An essential lynchpin in all of the studies cited above is the reliance on high extracellular concentrations of the pH buffer HEPES to increase the buffering capacity of the extracellular fluid and reduce or eliminate signs of feedback inhibition. An important underlying assumption in these studies is that extracellular HEPES reduces changes in extracellular pH without having any other effects on cells. However, Fahrenfort et al. (2009) and Trenholm and Baldridge (2010) have shown that increases
in extracellular HEPES induce an intracellular acidification in horizontal cells, and it is well established that intracellular acidification can reduce calcium flux through voltage-gated calcium channels (cf. Dixon et al. 1993), one of the key hallmarks of feedback inhibition onto photoreceptors. It is possible that high external HEPES results in the intracellular acidification of photoreceptors, bipolar cells, and glial cells as well, resulting in the potential modulation of a large range of channels and transporters. Thus the effects of high extracellular HEPES may be quite complex and difficult to interpret and are not likely to be limited to simply buffering free levels of H+ in the extracellular milieu. As noted by Fahrenfort et al. (2009), this observation weakens significantly the strength of the data obtained thus far in support of the proton hypothesis of lateral inhibition.

Despite the caveat that microdomains of pH regulation may exist in the intact retina, our current and past results are more consistent with the hypothesis that alkalization induced by depolarization of horizontal cells might relieve inhibition induced by protons released by photoreceptors themselves via exocytosis. DeVries (2001) reported that the fusion of photoreceptor synaptic vesicles with the plasma membrane induces a short-term inhibition of neurotransmitter release from those same photoreceptors due to the block of calcium channels by H+ coreleased with neurotransmitter. A similar inhibition of photoreceptor calcium channels by protons exocytosed from cone photoreceptors of the newt was reported by Hosoi et al. (2005). The cone photoreceptor synapse is likely to be held in a relatively acidic state by this process when photoreceptors are depolarized due to tonic release of glutamate and protons in tandem. Depolarization of horizontal cells by the photoreceptor neurotransmitter could facilitate the removal of these protons from the extracellular space, thus liberating the photoreceptors from self-induced inhibition. Given the widespread presence of high levels of acidity in synaptic vesicles, which is believed to be key in enabling vesicles to be loaded with neurotransmitter (cf. Chaudhry et al. 2008 for review), we speculate that this may be a common feature of synaptic signaling. We hypothesize that extracellular acidification due to the corelease of protons with neurotransmitter when presynaptic vesicles fuse at synapses throughout the nervous system may be tempered by postsynaptic elements that act to relieve the H+ block of calcium channels and resestinate the synapses for further release of neurotransmitter.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


