Published July 30, 2007

Modulation of Extracellular Proton Fluxes from Retinal Horizontal Cells of the Catfish by Depolarization and Glutamate

Matthew A. Kreitzer,[1] Leon P. Collis,[2] Anthony J.A. Molina,[3,4] Peter J.S. Smith,[2] and Robert Paul Malchow[5,6]

[1]Department of Biology, Indiana Wesleyan University, Marion, IN 46953
[2]BioCurrents Research Center, Program in Molecular Physiology, Marine Biological Laboratory, Woods Hole, MA 02543
[3]Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, Boston, MA 02111
[4]Obesity Research Center, Evans Department of Medicine, Boston University School of Medicine, Boston, MA 02111
[5]Department of Biological Sciences and Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, IL 60607
[6]Division of Integrative Organismal Systems, National Science Foundation, Arlington, VA 22230

Self-referencing H^+-selective microelectrodes were used to measure extracellular proton fluxes from cone-driven horizontal cells isolated from the retina of the catfish (*Ictalurus punctatus*). The neurotransmitter glutamate induced an alkalinization of the area adjacent to the external face of the cell membrane. The effect of glutamate occurred regardless of whether the external solution was buffered with 1 mM HEPES, 3 mM phosphate, or 24 mM bicarbonate. The AMPA/kainate receptor agonist kainate and the NMDA receptor agonist *N*-methyl-D-aspartate both mimicked the effect of glutamate. The effect of kainate on proton flux was inhibited by the AMPA/kainate receptor blocker CNQX, and the effect of NMDA was abolished by the NMDA receptor antagonist DAP-5. Metabotropic glutamate receptor agonists produced no alteration in proton fluxes from horizontal cells. Depolarization of cells either by increasing extracellular potassium or directly by voltage clamp also produced an alkalinization adjacent to the cell membrane. The effects of depolarization on proton flux were blocked by 10 μM nifedipine, an inhibitor of L-type calcium channels. The plasmalemma Ca^{2+}/H^+ ATPase (PMCA) blocker 5(6)-carboxyeosin also significantly reduced proton flux modulation by glutamate. Our results are consistent with the hypothesis that glutamate-induced extracellular alkalinizations arise from activation of the PMCA pump following increased intracellular calcium entry into cells. This process might help to relieve suppression of photoreceptor neurotransmitter release that results from exocytosed protons from photoreceptor synaptic terminals. Our findings argue strongly against the hypothesis that protons released by horizontal cells act as the inhibitory feedback neurotransmitter that creates the surround portion of the receptive fields of retinal neurons.

INTRODUCTION

Signals generated within vertebrate photoreceptors are passed to second order neurons within the outer plexiform layer, where the synaptic terminals of the photoreceptors make contacts with horizontal and bipolar cells (Kolb et al., 2001). It is at this level of the retina that visual signals are transformed into the classic center-surround receptive fields characteristic of retinal neurons (Werblin and Dowling, 1969).

Debate persists as to the molecular mechanisms used within the outer plexiform layer to establish the surround portion of these receptive fields. One hypothesis currently under consideration suggests that protons may be the key agents involved in the establishment of lateral inhibition in the outer retina. A lynchpin underlying this hypothesis is the remarkable sensitivity of synaptic transmission within the outer retina to small changes in extracellular pH. Kleinschmidt (1991) demonstrated that altering the extracellular solution from a pH of 7.8 to 7.5 resulted in a suppression of the light-induced signal in second order neurons by nearly two thirds, and reducing the external pH to 7.2 virtually abolished synaptic transmission. A high sensitivity of retinal signals to changes in extracellular pH was also reported by Barnes et al. (1993) and Harsanyi and Mangel (1993). Barnes and coworkers provided strong evidence that this modulation was due to the high sensitivity of photoreceptor calcium channels to extracellular hydrogen ions. Increases in the extracellular concentration of hydrogen ions (H^+) shift the voltage-activation curve of the calcium conductance of photoreceptors to more depolarized levels and also reduce the absolute magnitude of the calcium conductance, leading to a decrease in calcium-dependent neurotransmitter release from these cells. Thus, one plausible mechanism by which horizontal cells could induce tonic inhibition of photoreceptors in the dark would be by increasing the extracellular concentration of protons, acidifying the extracellular milieu,

Correspondence to M.A. Kreitzer: matthew.kreitzer@indwes.edu

Abbreviations used in this paper: DAP-5, 2-amino-5-phosphonovaleric acid; PMCA, plasmalemma Ca^{2+}/H^+ ATPase.

Published July 30, 2007

which would act to close calcium channels in the photoreceptor synaptic terminals and inhibit neurotransmitter release.

Recent work by Hirasawa and Kaneko (2003), Vessey et al. (2005), and Cadetti and Thoreson (2006) has lent additional support to the H^+ hypothesis of lateral inhibition. Using the retina of the newt, Hirasawa and Kaneko (2003) reported that enriching the pH-buffering capacity of the extracellular solution reduced inhibition as defined by shifts in the voltage dependence of calcium currents of photoreceptors. Vessey et al. (2005) reported that high levels of the pH buffer HEPES reduced both rollback and the depolarization to red light of the electrical responses of goldfish horizontal cells, features which have been attributed to feedback from horizontal cells to the photoreceptors. Cadetti and Thoreson (2006) found that direct hyperpolarization of tiger salamander horizontal cells induced a shift of the calcium current of cones to more negative voltages and increased its amplitude, and further noted that both effects were abolished by enhancing the extracellular pH buffering capacity with high concentrations of HEPES.

Should the H^+ hypothesis of lateral feedback inhibition prove to be correct, it could significantly alter our view of how neuronal receptive fields are established in the retina, and by extension to the processing of inhibitory signals throughout the nervous system. Neuronal activity in many areas of the nervous system has been shown to be associated with changes in extracellular pH (compare Deitmer and Rose, 1996; Chesler, 1990, 2003). Viewed by many in past years as an epiphenomenon of little consequence, the H^+ hypothesis of lateral feedback inhibition suggests that such changes in extracellular pH might be of fundamental importance to shaping the responses of neurons to stimuli.

If the H^+ hypothesis of lateral feedback inhibition is correct, then depolarization of horizontal cells should lead to an increase in the extracellular concentration of H^+ in the synaptic cleft—the space in the synaptic cleft should become more acidic, with consequent inhibition of calcium channels on photoreceptors. To test the H^+ hypothesis of surround inhibition, Molina et al. (2004) used self-referencing H^+-selective electrodes to record H^+ fluxes from isolated horizontal cells of the skate retina maintained in primary culture. The H^+ hypothesis of surround inhibition would suggest that glutamate, the neurotransmitter believed to be released by photoreceptors (Copenhagen and Jahr, 1989), should depolarize the horizontal cells and produce an increase in the release of protons, leading to an acidification adjacent to the cell membrane. However, Molina et al. found the opposite; glutamate consistently produced a marked alkalinization of the extracellular fluid near the membrane of the horizontal cell.

What could account for the difference between the observations of Molina et al. arguing against a role for H^+ in feedback inhibition and those cited above in favor of the H^+ hypothesis? Hirasawa and Kaneko (2003) examined responses of cone-driven cells of salamander. Vessey et al. (2005) focused on the cone-driven responses of zebrafish and goldfish. The initial observations of inhibitory feedback from horizontal cells onto photoreceptors were done on cone photoreceptors of the turtle (Baylor et al., 1971). Cadetti and Thoreson (2006) also examined feedback from horizontal cells onto cone photoreceptors. Inhibitory feedback onto cone photoreceptors has also been well documented in goldfish, carp, perch, catfish, and tiger salamander (for review see Wu, 1992). In contrast, the work of Molina et al. (2004) used the all rod-retina of the skate. In this species, there are no cone photoreceptors, and only a single class of rods (Szamier and Ripps, 1983). Thus, by definition, the horizontal cells in the skate are rod-driven horizontal cells. There is a marked paucity in the literature relating to inhibitory feedback onto rod photoreceptors as compared with the wealth of studies describing feedback inhibition onto cones, and indeed, it has been suggested that feedback from horizontal cells to rods does not normally occur.

To address this issue, we have examined proton fluxes from horizontal cells isolated from the retina of the catfish. Two types of horizontal cells are present and easily identified in this retina: a cone-driven horizontal cell and a rod-driven horizontal cell. Using self-referencing H^+-selective electrodes, we have monitored glutamate-induced alterations in extracellular proton fluxes from both cone-and rod-driven horizontal cells of this species. We find that glutamate consistently induces an alkalinization of the solution adjacent to the cell membranes of both cell types, and our findings are consistent with the hypothesis that the glutamate-induced alkalinizations result from calcium entry onto cells with subsequent activation of the plasmalemma Ca^{2+}/H^+ ATPase (PMCA). Our results argue strongly against the H^+ hypothesis of lateral inhibition in the outer retina.

MATERIALS AND METHODS

Cell Dissociation

Channel catfish (*Ictalurus puctatus*) 3–12 inches in length were obtained from Osage Catfisheries and Kurtz's Fish Hatchery. Fish were maintained on a 12-h day/night light cycle at room temperature for up to 2 mo, and were dark adapted for at least 2 h before use. Catfish were anaesthetized with 1 g/gal (~0.26 g/liter) MS 222 (tricaine, Sigma-Aldrich) for 5–10 min, and then cervically transected and pithed. Eyes were enucleated, cut in half, and the posterior portion containing the retina was immersed in a solution of modified Leibowitz culture medium (DeVries and Schwartz, 1989) containing 2 mg/ml papain and 1 mg/ml cysteine for 5 min. Retinas were then peeled off the back of the eye and placed in papain/cysteine-containing modified L-15 for 20–25 min and then washed eight times in modified culture medium lacking papain/cysteine, and mechanically agitated through a 5-ml graduated glass pipette. The cellular suspension (1–2 drops)

Published July 30, 2007

was placed in 35-mm culture dishes (Falcon 3001) preloaded with 3 ml modified culture medium. Recordings were made from cells whose nearest neighbors were at least 1 mm distant. Dishes were maintained at 14°C for up to 3 d. 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 Molina et al. (2004) (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 pH 7.50 with KOH. The pipette tip was placed in contact with a highly selective H^+-selective resin (hydrogen ionophore 1-cocktail B; Fluka Chemica) and ~30 μm of resin drawn up. The resin used in these experiments has a high degree of selectivity for H^+ as compared with other ions, and is reported to be $>10^9$ times more sensitive to H^+ than to Na^+ or K^+ (Fluka, 1996). It is unlikely that extracellular voltage fields associated with cellular currents contribute to the signals we report here, as such fields are usually in the nanovolt range and below the sensitivity of ion-selective self-referencing probes (Kuhtreiber and Jaffe, 1990; Smith et al. 1999, 2007). Electrical potentials arising from local boundary conditions associated with membrane surface charges (McLaughlin et al., 1971, 1981) are also unlikely to be the source of the signals we report. Such fields typically drop away with the Debye length and do not extend into the medium by more than tens of angstroms (compare Cevc, 1990; Hille, 1992). Our sensors were located at least 1 μm away from the cell surface. Moreover, the measured gradients extended many tens of micrometers away from the cell.

Self-referencing Recordings

H^+-selective microelectrodes were used in a self-referencing mode (Smith and Trimarchi, 2001; Smith et al., 2007). In this format, the electrode is first placed adjacent to the membrane of a cell, and a reading taken; the electrode is then moved a set distance away and a second reading taken. By subtracting the signals from the two different points, a differential signal is obtained that reflects the difference in proton concentration at the two locations. This process results in the elimination of much of the slow electrical drift inherent in the signal from such electrodes, since that drift is common to the two positions measured. 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 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 the present 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. Electronics, software, and mechanical control of electrode movement were the same as described in Molina et al. (2004), and were the products of the BioCurrents Research Center. Electrodes were calibrated using commercially purchased pH standards: pH 6.00, 7.00, and 8.00 (SB104-1, SB108-1, and SB112-1, respectively; Fisher Scientific). Only electrodes possessing Nernst slopes between 45 and 60 mV (pH unit)$^{-1}$ were used.

Experimental Protocol

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 probably be significantly disturbed or eliminated by rapid superfusion of solutions around the cell. Consequently, we applied solutions by adding 1 ml of solution by a handheld pipette and allowing the solution to settle. A typical experiment began by replacing the culture medium completely with solution containing 1 mM HEPES; the final volume of fluid in the dish was set to 2 ml. The extracellular solution used in most experiments consisted of (in mM) NaCl 126, KCl 4, $CaCl_2$ 3, $MgCl_2$ 1, HEPES 1, glucose 15; pH was adjusted to 7.40 with NaOH (all chemicals were purchased from Sigma-Aldrich unless otherwise indicated). After locating a cell, the H^+-selective electrode was placed ~1–2 μm from the cell membrane. Differential extracellular recordings were made for several minutes to ensure a steady baseline reading. Normal extracellular solution (1 ml) 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. Some time (usually several minutes) later, 1 ml of 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. 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. Doses chosen for kainate, NMDA, and other drugs were based on previously published work on the effects of these drugs on catfish cone horizontal cells (compare O'Dell, 1989; Gafka and Linn, 2001; Davis and Linn, 2003) or on our own previous experiences with these compounds on isolated horizontal cells of the skate. In experiments examining the effects of *N*-methyl-D-aspartate, $MgCl_2$ was omitted from the solutions. In experiments using 5-(and-6)-carboxyeosin diacetate, succinimidyl ester (Invitrogen; and hereafter referred to as carboxyeosin), a stock solution of 10 mM was prepared in DMSO. Cells were incubated in 10 μM of the drug (stock solution diluted in normal catfish modified L-15 solution) for 30 min and then washed three times with catfish modified L-15 solution. Responses from cells were examined in normal catfish Ringer's solution 15 min to 3 h after treatment. Caloxin 1b1 was synthesized by Dalton Chemical Laboratories Inc. The peptide is as follows: TAWSEVLH-LLSRGGG-OH, and was stored at −20°C.

We conducted separate control experiments to ensure that drugs did not alter the ability of H^+-selective electrodes to sense changes in pH. Two types of control experiments were performed. First, proton gradients were measured from H^+ source pipettes (Molina et al., 2004) 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 then presence of the drug. In these control experiments, it was noted that the L-type calcium channel blocker nifedipine, prepared as a 1 mM stock solution in DMSO and then diluted in catfish Ringer's solution, produced a progressive loss of sensitivity of the H^+-selective electrodes to protons at concentrations of 100 μM and greater. This progressive decrease in sensitivity developed more rapidly with higher concentrations of the drug. However, 10 μM nifedipine produced at most only a modest decline in sensitivity of the H^+-selective sensors during the typical time course of the experiments. The calibration values for electrodes in 10 μM nifedipine were not statistically different from those in control Ringer's solution, and represented at most a 5% decrease in sensitivity of the sensors over the period of 1 h. All other compounds used were found to be without effect on H^+ sensors at the concentrations indicated. Additional control experiments demonstrated that addition of saline containing DMSO at the concentrations used to prepare 10 μM nifedipine did not by itself influence either the characteristics of the H^+-selective electrodes or proton flux measured directly from the horizontal cells.

Published July 30, 2007

In experiments examining H^+ flux in PBS, the 1 mM HEPES was replaced by 0.22 mM NaH_2PO_4 and 2.78 mM Na_2HPO_4, which resulted in the solution having a pH of 7.4. In experiments examining proton flux in bicarbonate-buffered solutions, the 1 mM HEPES was removed, NaCl was reduced to 102 mM, and 24 mM $NaHCO_3$ was added to the solution. The bicarbonate solution was bubbled with 5% CO_2/95% air for 20 min before use, and this same gaseous mixture was blown continuously over the 35-mm culture dishes containing the cells during the experiment.

Voltage Clamp

In experiments examining proton fluxes under voltage clamp, catfish cells were clamped using an AxoClamp 2B amplifier (Axon Instruments) operating in the discontinuous single electrode voltage clamp (dSEVC) mode. Conventional sharp high resistance (35–50 MΩ) electrodes were filled with 100 mM KCl. pClamp ver. 8.0 and a 1322A Digidata system (Axon Instruments) were used to control the voltage of the cells when conducting voltage-step protocols to examine the quality of the clamp. The ground wire in the bath was connected to the head stages of both the self-referencing system and the AxoClamp 2B amplifier. This configuration increased the level of background electrical noise in the self-referencing system. When self-referencing H^+ recordings were being made, the pClamp software was turned off, and the voltage changed manually using the AxoClamp 2B.

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 presented throughout as the mean ± SEM. For most experiments, values reported reflect the average of the 30 points before application of a drug, the 30 points after application, and the 30 points some period of time later, usually 300 s after drug application.

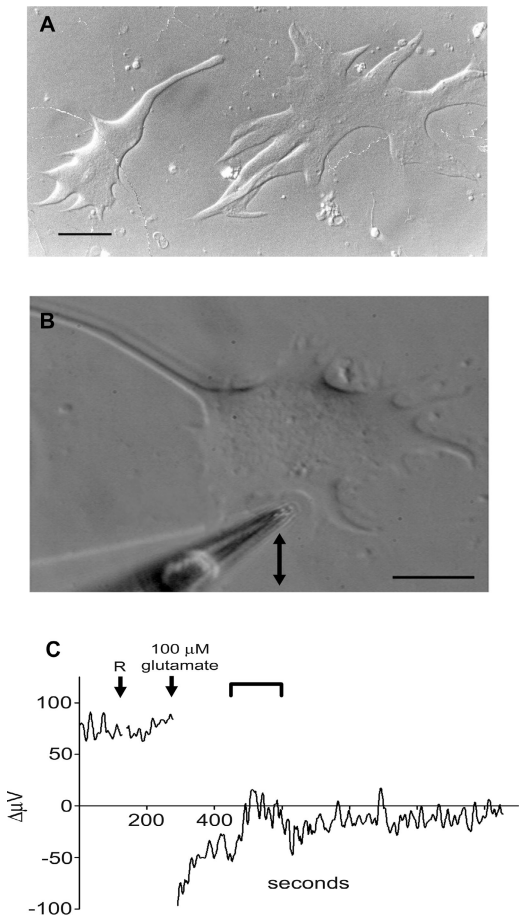

Figure 1. (A) Photomicrograph of a cone (left) and rod (right) horizontal cell isolated from the retina of the catfish. Bar, 25 μm. (B) A cone horizontal cell shown with an H^+-selective microelectrode positioned several microns away from the cell membrane. Differential recordings were made by laterally translating the electrode to a position 30 μm distant from the cell and computing the difference in signal between the near and far poles of the recording. The double arrow indicates the movement from near to far positions. Bar, 25 μm. (C) Self-referencing recording from one catfish cone horizontal cell. The initial trace was obtained with the cell bathed in 2 ml normal catfish Ringer's solution. At the first arrow (marked "R"), 1 ml additional catfish Ringer's solution was added; the portion of the trace during which artifacts from solution addition occurred has been subtracted from this trace and all subsequent figures. At the second arrow, an additional 1 ml of catfish Ringer's solution containing 400 μM glutamate was added to the dish, bringing the total concentration of glutamate in the chamber to 100 μM. At the beginning of the bar, the electrode was moved 400 μm away from the cell and a differential trace recorded in this control location for ~120 s. At the end of the bar, the electrode was repositioned so that the electrode at the near pole was again 1–2 μm away from the cell.

RESULTS

Enzymatic dissociation of the retina of the channel catfish results in plentiful numbers of two large cells previously identified as horizontal cells. Fig. 1 A shows a photomicrograph of these two types of cells, with what has previously been characterized as a cone-driven horizontal cell shown on the left and the much larger rod horizontal cell portrayed on the right (Dong et al., 1994). Self-referencing H^+ recordings from these cells were made with electrodes first placed within 1–2 μm of the cell membrane (Fig. 1 B) and then when the electrode was translated some 30 μm distant; subtracting the signals from the two points produced a differential signal reflecting the difference in proton concentration at the two locations. Fig. 1 C shows the differential self-referencing signal recorded from one cone horizontal cell bathed first in catfish Ringer's solution containing 1 mM of the pH buffering agent HEPES. A standing positive signal, in this case ~70 μV, was detected. The fact that the signal was positive indicated that the concentration of protons was higher next to the cell than in the solution some 30 μm away. Recordings from other cells showed that, as expected, the absolute magnitude of the standing proton flux varied as a function of the distance of the H^+-selective electrode away from the cell, becoming smaller as the electrode was moved further from the cell (compare Molina et al., 2004).

Glutamate Alters Cone Horizontal Cell H^+ Fluxes

Fig. 1 C also shows the effect of glutamate on H+ fluxes from these cells. At the first arrow, a control bolus of 1 ml catfish Ringer's solution was applied by hand pipette

Published July 30, 2007

to the dish. At the second arrow, 1 ml of a catfish Ringer's solution containing 400 μM glutamate was added to the dish, resulting in a final concentration of 100 μM glutamate in the chamber. The pH of the glutamate solution had previously been adjusted to match the pH of the normal catfish Ringer's solution. Addition of glutamate to the bath consistently led to a marked drop in the self-referencing signal, and indeed the differential response usually became negative, indicating that the solution adjacent to the cell membrane was now more alkaline than the solution some 30 μm away. Also typical was a slow period of partial decline in the size of the negative response. At ~500 s in this trace, the self-referencing electrode was moved vertically to a position 400 μm away from the cell and differential measurements taken (indicated by the bar above the trace). Under these conditions, the H^+ concentration at the two points (400 and 430 μm) away from the cell should be the same, and the differential reading should thus be near zero. This type of control reading was also done for every cell during the course of an experiment. The electrode was then returned to a position such that the near pole of recording was again 1–2 μm away from the cell (end of bar above trace, ~625 s). As can be seen, with glutamate still present in the bath, the self-referencing signal remained significantly below the value obtained initially at the start of the experiment.

In 11 cone horizontal cells examined in this fashion, the average standing signal from the self-referencing electrodes was found to be 65 ± 9 μV; following the addition of plain catfish Ringer's solution the signal was 74 ± 11 μV, a value not statistically different from before addition of solution. The average value for the self-referencing signal during the first 30 s after application of 100 μM glutamate was found to be −69 ± 13 μV; after 300 s, with 100 μM glutamate still present in the bath, the differential signal averaged −25 ± 13 μV. The values at both time points were statistically significantly different from the value for the standing proton flux before the application of glutamate.

Glutamate Receptor Subtypes and Modulation of H^+ Flux

Cone catfish horizontal cells are known to possess both AMPA/kainate and NMDA-type ionotropic glutamate receptors (O'Dell and Christensen, 1989). We looked to see if selective activation of these types of receptors were capable of eliciting changes in proton flux similar to that observed for glutamate. Fig. 2 A shows the response of one catfish cone horizontal cell to the application of kainate, an agent known to activate AMPA/kainate-type receptors. The standing proton flux is first seen, and the application at the first arrow of 1 ml control Ringer's solution again did not significantly alter the standing differential signal. Addition of kainate to a final concentration of 20 μM (second arrow) produced a significant alteration in the signal, which again became negative and remained so throughout the experiment. At the time indicated by the bar above the trace, the electrode was moved to a position some 400 μm from the cell; at this control location, the differential recording that was obtained was close to 0 μV, as expected. The electrode was then moved back to its original position relative to the cell. In seven cells, the standing signal before kainate application was 48 ± 5 μV; following kainate application, the signal became negative, with the self-referencing signal averaging −58 ± 8 μV after the first 30 s of application and settling to −32 ± 7 μV after 300 s. Fig. 2 B shows that the effect of kainate on proton flux could be abolished when the dish contained 100 μM of the AMPA/kainate receptor blocker, CNQX. In seven cells bathed in 100 μM CNQX, the signal averaged 33 ± 5 μV before application of 20 μM kainate and 37 ± 4 μV after the addition of 20 μM kainate, values that were not statistically significantly different. 100 μM CNQX by itself did not cause any significant alteration in the basal proton flux from the catfish cone horizontal cells.

Fig. 2 C shows the response of one cell to the application of 300 μM NMDA and illustrates that NMDA also produced a significant alteration in proton flux. The average self-referencing signal before addition of NMDA was 98 ± 5 μV ($n = 8$); after the application of NMDA, the self-referencing signal was initially altered to an average value of −85 ± 16 μV over the first 30 s and −32 ± 6 μV after 300 s. This response to NMDA was blocked by prior incubation of the cells with 100 μM 2-amino-5-phosphonovaleric acid (DAP-5), an agent known to competitively block the opening of NMDA receptors in retinal neurons (Coleman and Miller, 1988) (Fig. 2 D). In six cells bathed in 100 μM DAP-5, the initial signal averaged 99 ± 6 μV before application of 300 μM NMDA and 95 ± 5 μV after the addition of 300 μM NMDA, and was not statistically significantly different. DAP-5 did not by itself induce any significant alteration in the standing proton flux. In this respect, the effects of NMDA on proton flux from catfish cone horizontal cells differ from those of skate, in that NMDA did not alter proton flux from skate horizontal cells, an observation consistent with the absence of NMDA receptors in the skate horizontal cells (Kreitzer et al., 2003).

Catfish cone horizontal cells possess Group 1 and Group 3 metabotropic receptors (Gafka et al., 1999). We consequently applied DHPG, known to activate Group 1 metabotropic receptors, and LAP-4, an agonist of Group 3 receptors (Caramelo et al., 1999), to determine if activation of metabotropic receptors could similarly alter proton fluxes in these cells. Fig. 2 (E and F) shows that neither DHPG nor LAP-4 at 100 μM induced a significant alteration in the signal from the H^+-selective electrode. The standing signal from seven cells before the application of DHPG was 56 ± 7 μV; after addition of 100 μM DHPG to the bath, the signal was 58 ± 5 μV.

Published July 30, 2007

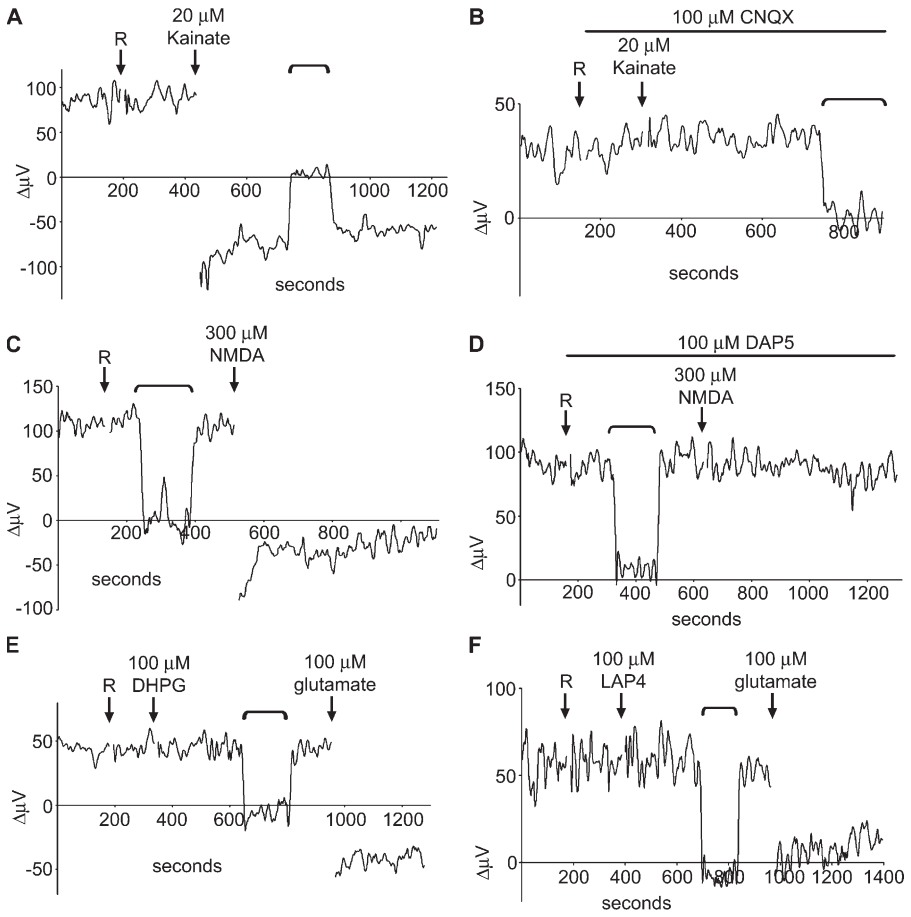

Figure 2. Effects of glutamate receptor agonists on proton fluxes measured from individual catfish cone horizontal cells. In all six traces, the first arrow indicates the addition of 1 ml control solution, and the second arrow indicates the application of either ionotropic or metabotropic glutamate receptor agonists. Downward-hooked bars above traces indicate differential recordings obtained with the electrode 400 μm distant from the cell. (A) Addition of kainate to a final concentration of 20 μM produced a large alteration in the self-referencing signal. (B) Addition of 20 μM kainate caused no significant alteration in proton flux when 100 μM of the AMPA-receptor blocker CNQX was present in the bath. (C) 300 μM NMDA applied to catfish cone horizontal cells induced a significant alteration in the signal from the self-referencing H^+ selective electrodes. (D) Addition of NMDA caused no significant alteration in proton flux when 100 μM of the NMDA receptor blocker DAP-5 was also present in the solution. (E) Addition of 100 μM of the Group 1 metabotropic glutamate receptor agonist DHPG did not alter proton flux. However, addition of 100 μM glutamate (third arrow) did promote a significant alteration in proton flux from the same cell. (F) Addition of 100 μM of the Group 3 metabotropic agonist LAP-4 did not alter proton flux. However, addition of 100 μM glutamate (third arrow) did promote a significant alteration in proton flux from the same cell.

The proton signal from an additional seven cells measured before the application of 100 μM LAP-4 was 45 ± 5 μV; the signal after application of the drug was 42 ± 6 μV. Application of 100 μM glutamate led to a statistically significant alteration in proton flux measured from the same cells. Thus, activation of metabotropic glutamate receptors does not appear to directly alter the proton flux measured from the horizontal cells.

The Role of Calcium in the Modulation of H^+ Flux by Glutamate

Glutamate application produces a significant rise in intracellular calcium in catfish cone horizontal cells through multiple mechanisms (Linn and Christensen, 1992). This elevated intracellular calcium is likely to be removed from the cell in part by the action of the PMCA pump. The PMCA pump operates by taking protons into the cell from the extracellular space when calcium ions are extruded from the interior of the cell (Schwiening et al., 1993; Hao et al., 1994; Salvador et al., 1998). If the changes in proton flux induced by glutamate result from activation of the PMCA pump, then removal of extracellular calcium should reduce or eliminate this effect; application of glutamate in a Ringer's solution with 0 mM extracellular calcium should prevent the calcium load and eliminate activation of the PMCA pump, while still allowing glutamate to depolarize the cells. As shown in Fig. 3 A, removal of extracellular calcium did indeed significantly reduce the ability of glutamate to modulate proton flux from catfish cone horizontal cells. A standing proton flux was still present, and glutamate no longer induced a significant alteration in the proton flux. The average self-referencing signal observed from five cells bathed in 0 mM Ca^{2+} Ringer's solution was 41 ± 4 μV before and 28 ± 5 μV after the addition of 100 μM glutamate, values which were not statistically significantly different. In separate experiments, we found that 100 μM glutamate still produced a depolarization of cells when bathed in 0 mM calcium Ringer's solution. The average resting potential of the cells in 0 mM calcium was −75 ± 8 mV, and the voltage of the cells after the addition of 100 μM glutamate averaged −12 ± 4 mV.

Both the AMPA/kainate and NMDA glutamate receptors of catfish horizontal cells are permeable to calcium

Published July 30, 2007

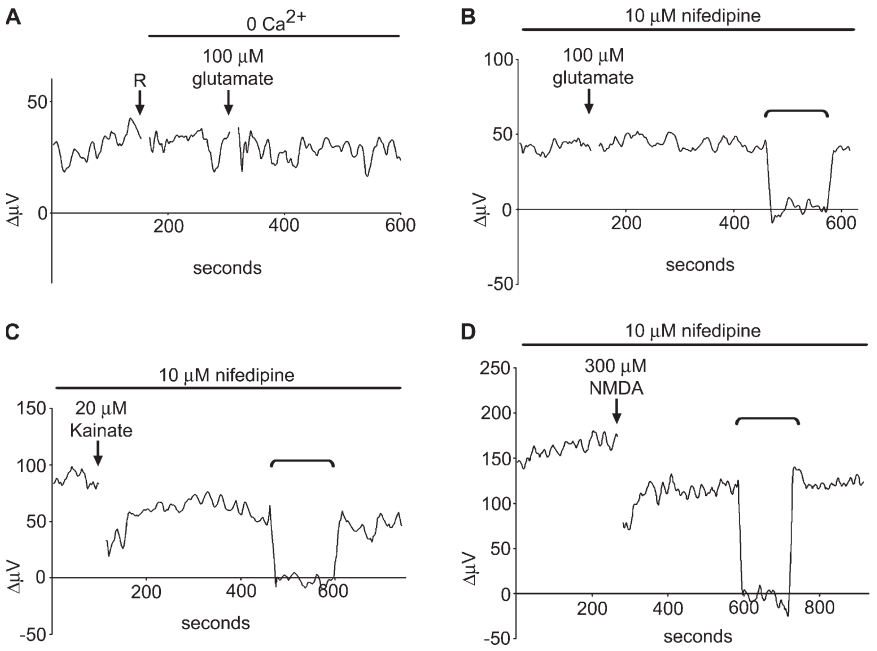

Figure 3. (A) Elimination of extracellular calcium significantly reduced the ability of 100 μM glutamate to alter proton fluxes from catfish cone horizontal cells. In this experiment, the cell was continuously bathed in a Ringer's solution lacking in added calcium and which also had 1 mM EGTA present in the bath. The first arrow indicates application of 1 ml additional 0 mM calcium Ringer's solution, and the second arrow indicates addition of 1 ml 0 mM calcium Ringer's solution plus glutamate, bringing the final concentration of glutamate in the bath to 100 μM. (B) Effects of the L-type calcium channel blocker nifedipine on glutamate-induced proton fluxes. The trace shows a recording from a single cell continuously bathed in catfish Ringer's solution containing 10 μM nifedipine. 100 μM glutamate in 10 μM nifedipine Ringer's solution was added at the arrow. Nifedipine greatly reduced the ability of glutamate to alter proton flux. (C) Response of a different cone horizontal cell to kainate with the cell bathed in 10 μM nifedipine. The arrow shows the response induced upon the addition of 20 μM kainate. The response to kainate was consistently smaller in the presence of 10 μM nifedipine as compared with that in normal catfish Ringer's solution. (D) Response of a different cone horizontal cell to application of 300 μM NMDA (arrow) in the presence of 10 μM nifedipine.

(Linn and Christensen 1992). Activation of these receptors also leads to depolarization of the cell and activation of L-type calcium channels, which will lead to a further increase in the influx of calcium into the cell (Shingai and Christensen, 1986). To determine the relative importance of calcium influx through voltage-gated calcium channels as compared with the glutamate-gated channels in the glutamate-induced modulation of proton flux, we examined the responses of cells to glutamate when bathed in solutions containing 10 μM nifedipine. Fig. 3 B shows that this concentration of nifedipine induced a nearly complete block of the effects of glutamate on proton flux. In seven cells, the average self-referencing signal from cells bathed in 10 μM nifedipine was 41 ± 7 μV before glutamate application. The average response for the 30 s following application of 100 μM glutamate was 31 ± 6 μV and 46 ± 6 μV after 300 s. Thus, it appears that calcium flux through the L-type calcium channels is the major determinant of the changes in proton flux that are induced by glutamate. We also examined the effects of nifedipine on the kainate-induced changes in proton flux (Fig. 3 C). Kainate was able to induce an alteration in proton flux, but the size of the alteration was typically smaller than that obtained without nifedipine present. The average size of the self-referencing signal from seven cells bathed in 10 μM nifedipine was 67 ± 7 μV before addition of kainate. The peak decrease in proton flux following the addition of 100 μM kainate was 53 ± 4 μV, significantly less than the decrease to −58 μV without nifedipine present, and the proton flux signal typically did not go below zero. Experiments examining the effect of NMDA in the presence of nifedipine produced similar results (Fig. 3 D). The response from seven cells before adding NMDA but bathed in 10 μM nifedipine was 119 ± 16 μV, and the proton flux decreased to 102 ± 7 μV in the 30 s following the application of NMDA, a significantly smaller alteration than the change to −85 μV found in control Ringer's solution.

The Effect of Depolarization on Extracellular H^+ Flux

The observation that removal of extracellular calcium eliminates the change in proton flux suggests that simple depolarization of the horizontal cells, which should activate L-type calcium channels, should also promote a significant change in proton flux in the horizontal cells. To test this hypothesis, we first examined changes in the self-referencing differential signal upon the addition of 50 mM potassium, which should lead to a depolarization of cells due to the alteration of the Nernst potential for potassium. Fig. 4 A shows recordings from a single cone horizontal cell first in normal catfish Ringer's solution and following the addition of solution to bring the final concentration of potassium to 50 mM. Addition of potassium led to a significant depolarization of catfish cone horizontal cells; in four cells examined, the resting membrane potential before high potassium averaged −81 ± 2 mV, and the cells were depolarized to an average value of −14 ± 2 mV following the addition of high potassium Ringer's solution. Addition of potassium also led to a significant alteration in proton flux, with the area adjacent to the cell membrane once again

Published July 30, 2007

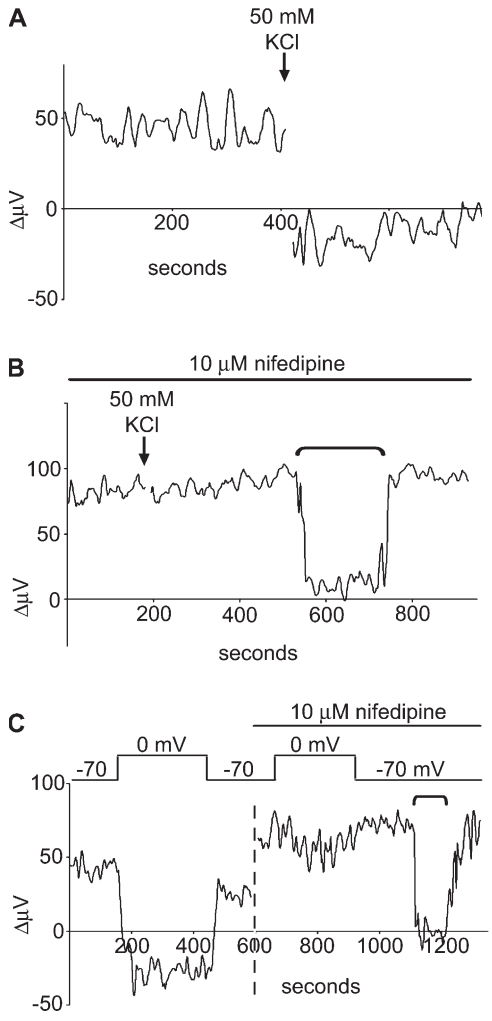

Figure 4. Depolarization of cone catfish horizontal cells induces an extracellular alkalinization. (A) Effects of increased extracellular potassium on proton flux measured from catfish cone horizontal cells. Recording from a single cell first in normal catfish Ringer's solution, followed by (arrow) exchange of the solution for one containing 50 mM potassium. (B) The effect of potassium was completely eliminated when 10 μM of the L-type calcium channel blocker nifedipine was present in the bath. The arrow again indicates the time at which the normal catfish Ringer's solution was replaced with one containing 50 mM potassium. The bar indicates a control recording with the electrode moved 400 μm away from the cell. (C) Direct depolarization of a voltage-clamped catfish cone horizontal cell also induces an extracellular alkalinization. The figure shows the self-referencing H^+ signal recorded from a single catfish cone horizontal cell voltage clamped first at −70 mV, then at 0 mV, and then upon return to −70 mV. At the dashed line, 10 μM nifedipine was added to the dish, and the voltage clamp procedure repeated: the cell was first held at −70 mV, then depolarized to 0 mV, and finally returned to −70 mV. The bar indicates recording with the self-referencing electrode positioned at a control location 400 μm away from the cell.

becoming more alkaline than the point 30 μm distant from the cell membrane. This observation was made in seven cells, which had a standing differential signal averaging 46 ± 7 μV before application of potassium; following the application of 50 mM potassium, the average signal for the first 30 s was −1 ± 5 μV, and −9 ± 4 μV after 300 s. Fig. 4 B shows that the effect of potassium on proton flux was eliminated when 10 μM of the L-type calcium channel blocker nifedipine was present in the bath. Under these conditions, with 10 μM nifedipine present in the dish, the standing proton flux before potassium was 72 ± 6 μV, and was 62 ± 8 μV in the 30 s following the exchange of the solution.

We also examined the effect of depolarization on proton flux under conditions in which horizontal cells were voltage clamped. Fig. 4 C shows a self-referencing recording obtained when one catfish cone horizontal cell was first voltage clamped to −70 mV; a standing proton flux signal of ∼45 μV was observed. Depolarizing the cell to 0 mV produced a rapid alteration in the proton flux signal, resulting in the solution near the cell becoming more alkaline than the solution 30 μm away. Switching the voltage back to −70 mV resulted in a rapid restoration of the proton flux to its original level. 10 μM nifedipine was then added to the bath and the experiment repeated. The standing flux was slightly larger when nifedipine was added to the bath and the cell still voltage clamped to −70 mV. Depolarization of the cells to 0 mV now produced only a very small alteration in proton flux. In nine catfish cone horizontal cells, the standing flux when voltage clamped at −70 mV was 55 ± 6 μV. When depolarized to 0 mV, the signal detected by the self-referencing electrode changed to −43 ± 6 μV; following the return of the cells to −70 mV, the signal returned to 51 ± 6 μV. In 10 μM nifedipine, cells voltage clamped at −70 mV produced a self referencing signal of 79 ± 9 μV. Jumping the voltage to 0 mV now resulted in a signal of 58 ± 11 μV.

The PMCA Blocker Carboxyeosin Reduces Glutamate Modulation of H^+ Flux

The data above are all consistent with the hypothesis that glutamate and simple direct depolarization promote an influx of intracellular calcium into the cells, which is then removed in part by the action of the PMCA pump, with consequent influx of protons into the cell from the extracellular solution. To further test this hypothesis, we examined the ability of carboxyeosin, a compound reported to block the activity of PMCA pumps in several preparations (Fierro et al., 1998; Choi and Eisner, 1999; Wanaverbecq et al., 2003), to inhibit the alterations in H^+ flux induced by glutamate. In these experiments, cells were first incubated for 30 min in the ester form of this compound to permit intracellular access; cells were then washed with catfish modified L-15, and then H^+ fluxes examined in normal catfish Ringer's solution. Fig. 5 shows that in seven control cells recorded during the same period but not treated with carboxyeosin, the basal H^+ flux averaged 46 μV, and the addition of glutamate altered H^+ flux to a value of −103 μV 30 s after

Published July 30, 2007

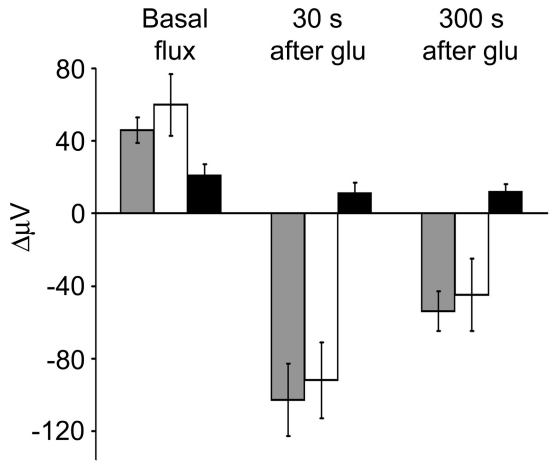

Figure 5. Effects of caloxin 1b1 and carboxyeosin on the ability of glutamate to induce alterations in H^+ flux. Gray bars show control responses from seven cells before the addition of glutamate (left), 30 s after addition of 100 μM glutamate (middle), and 300 s after addition of glutamate (right). White bars show the results obtained from four cells in the presence of 400 μM caloxin 1b1; there was no significant difference between the control responses and those obtained with caloxin 1b1 present. The black bars show the results obtained from five cells after having been bathed in 10 μM carboxyeosin. The standing flux was less, and the ability of glutamate to alter H^+ flux was significantly reduced as compared with control cells.

application and −54 μV 300 s after the application of glutamate. In contrast, the standing flux in five cells treated with carboxyeosin averaged 21 μV; addition of 100 μM glutamate now altered H^+ flux only to 11 μV 30 s after glutamate application and to 12 μV 300 s after glutamate application. Thus, there was a small but significant effect of carboxyeosin on basal H^+ flux, and a large reduction in the ability of glutamate to induce alterations in H^+ flux from the cells.

We also examined the effects of the peptide caloxin 1b1, which has been reported to block the activity of PMCA types 1 and 4 in mammalian aortic smooth muscle and endothelial cells (Pande et al., 2006). We found that this compound at a concentration of 400 μM did not significantly alter either the basal H^+ flux or the alteration in H^+ flux induced by glutamate. In four cells tested, we found that the basal flux before addition of caloxin was 60 ±17 μV; 30 s after addition of 100 μM glutamate, H^+ flux was altered to −92 ± 21 μV after 30 s and −45 ± 20 μV after 300 s, values that were statistically insignificantly different from the control cells cited above. We do not, however, take this observation as strong evidence against the PMCA hypothesis outlined above for several reasons. Caloxin 1b1 is a peptide, and as such might not act on the specific PMCA subtype in the catfish cone horizontal cells due to species and/or PMCA subtype differences. We presently do not know what PMCA subtype(s) are expressed by cone horizontal cells of the catfish or, indeed, any other species.

Glutamate Modulation of H^+ Fluxes in Phosphate and Bicarbonate pH Buffers

All of the data reported thus far were obtained with 1 mM of the pH buffer HEPES in the bath. Previous work examining proton fluxes from the rod-driven horizontal cells of the skate were also done with HEPES present in the medium (Molina et al., 2004). HEPES is not a natural buffering agent, and it has been reported that HEPES used alone to buffer extracellular pH in the intact retina can markedly alter the light-induced responses of horizontal cells of certain species (Hare and Owen, 1998; Hanitzsch and Kuppers, 2001). Consequently, we decided to examine proton fluxes from catfish cone horizontal cells in two other pH buffering situations. In the first condition, the extracellular pH was buffered using phosphate, using the amounts originally employed by Oakley and Wen (1989) in their study examining pH regulatory mechanisms in the retina of the toad. We replaced the 1 mM HEPES with 0.22 mM NaH_2PO_4 and 2.78 mM Na_2HPO_4, which produced a solution having a pH of 7.4. Fig. 6 A shows the response of one catfish cone horizontal cell to 100 μM glutamate when buffered with phosphate. In the phosphate-buffered Ringer's solution, a standing outward current was again observed, and application of an additional 1 ml solution containing glutamate (final concentration 100 μM) again produced a significant alteration in the self-referencing signal. The average differential signal from the H^+-selective probe was 71 ± 18 μV before the application of glutamate, and −25 ± 5 μV 30 s after the addition of 100 μM glutamate (five cells). Fig. 6 B shows that glutamate also produced a significant shift in proton flux when the medium was buffered using bicarbonate (24 mM) and 5% CO_2/95% air was continuously blown over the surface of the dish. Once again, a standing proton flux signal was detected, and application of glutamate resulted in a significant decrease in the level of acidity near the cell membrane. When buffered in bicarbonate, application of glutamate decreased the proton flux signal from 160 ± 25 μV to 32 ± 13 μV 30 s afterwards ($n = 6$).

Characteristics of the Basal H^+ Flux: Evidence for Na^+/H^+ Exchange Activity

Previous data obtained from skate horizontal cells suggested that the basal proton flux resulted from the activity of a sodium/hydrogen ion exchange mechanism (Molina et al., 2004). To see if this was also the case for the catfish cone horizontal cells, we examined fluxes when cells were bathed in a solution in which the 126 mM sodium normally present in the Ringer's solution had been replaced by 126 mM *N*-methyl-D-glucamine. We found that the basal proton flux recorded from five cone horizontal cells was significantly reduced in the 0 sodium solution, with the standing outward flux reduced to 1 ± 3 μV; the same cells had an averaged outward

Published July 30, 2007

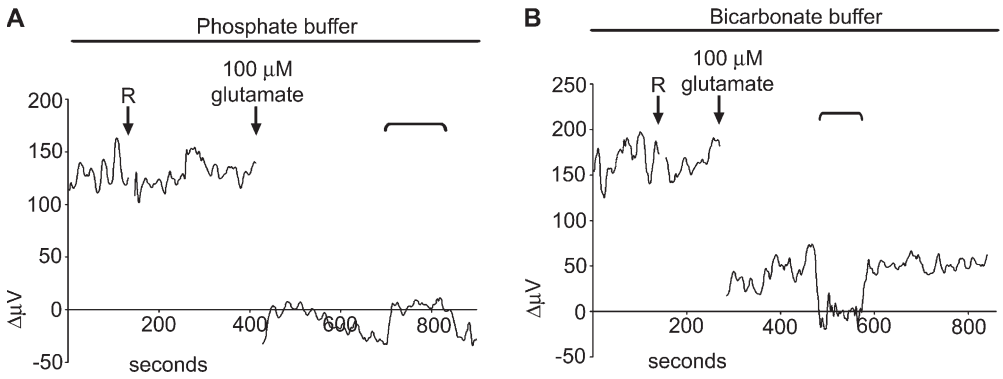

Figure 6. Proton flux signals in phosphate-buffered and bicarbonate-buffered solutions. (A) Self-referencing signal obtained from a single cone horizontal cell of the catfish bathed in phosphate-buffered Ringer's solution. Addition of 1 ml additional solution (first arrow) did not alter the standing signal. Application of 100 μM glutamate (second arrow), however, induced a marked alteration in the self-referencing signal. (B) Response from a single catfish cone horizontal cell when bathed in a solution buffered using bicarbonate (24 mM) and with 5% CO_2/95% air flowing across the surface of the dish. Once again, a standing proton flux signal is seen, and the application of 100 μM glutamate results in a significant decrease in the level of acidity near the membrane of the cell.

proton flux of 34 ± 2 μV in Ringer's solution containing normal sodium. Fig. 7 A shows an example of a recording obtained from one cell first in normal Ringer's solution and then in a solution in which the sodium had been replaced with *N*-methyl-D-glucamine. We also used pharmacological tools to examine the source of the standing H^+ signal. Fig. 7 B shows responses from one catfish cone horizontal cell to the application of 100 μM EIPA, a derivative of amiloride that has been reported to block the activity of Na^+/H^+ exchangers (Nguyen et al., 2000). In seven cells, EIPA significantly reduced the standing proton signal from 86 ± 12 to 2 ± 2 μV. These observations are similar to the effects observed previously on the standing proton flux of skate rod-driven horizontal cells (Molina et al. 2004) and suggests that Na^+/H^+ exchangers play an important role in the generation of the standing proton flux in catfish cone horizontal cells as well.

H^+ Fluxes from Rod Horizontal Cells of the Catfish

Finally, we also conducted experiments to examine some of the characteristics of proton fluxes from the rod horizontal cells of the catfish. We found that these cells also typically displayed a standing H^+ flux signal but that this signal was significantly smaller than that obtained from cone horizontal cells. The rod horizontal cells also responded to glutamate in the same general fashion as the cone horizontal cells, but the change in proton flux was again typically much smaller. Fig. 8 shows one such result. The initial portion of the trace shows the steady proton flux recorded first from a cone horizontal cell. The electrode was then repositioned to be adjacent to a nearby rod horizontal cell; a small standing signal is present. At the first arrow, 1 ml control catfish Ringer's solution was applied, which did not induce any significant change in the standing flux. When 100 μM glutamate was applied (second arrow), the flux became negative, indicating that the solution adjacent to the cell membrane was now more alkaline than the point 30 μm distant from the cell membrane. With the glutamate still present in the bath, the electrode was then moved back to the cone horizontal cell; the self-referencing signal for this cell had also become negative, but the change in response was significantly larger than that for the rod horizontal cell. In five rod horizontal cells examined, the standing flux was 10 ± 5 μV, compared with 63 ± 5 μV from five cone horizontal cells monitored in the same dishes. Upon the addition of 100 μM glutamate, the flux from rod horizontal cells was altered to a value of −8 ± 6 μV, compared with a drop to −31 ± 6 μV from the cone horizontal cells.

DISCUSSION

Our results demonstrate that the neurotransmitter glutamate induces an alkalinization of the solution directly adjacent to the plasma membrane of both cone and rod horizontal cells of the catfish. This glutamate-induced decrease in proton concentration is precisely opposite to the prediction made by the H^+ hypothesis of lateral inhibition; according to this hypothesis, addition of glutamate should result in an increase in the level of H^+ around the cell membrane. The observation that glutamate produces an alkalinization of the extracellular face in both cone and rod horizontal cells of the catfish, as well as the rod-driven horizontal cells of the skate (Molina et al., 2004), suggests that it is not the type of horizontal cell that is important in determining the nature of the response. Rather, this phenomenon is likely to be a general property of horizontal cells across species. Indeed, preliminary data obtained from isolated horizontal cells of the goldfish also indicate that glutamate produces an alkalinization of cone horizontal cells of that species as well (Malchow, R.P., S. Sartipi, Z. Tun, F. Iqbal, and P.J.S. Smith. 2004. Society for Neuroscience. Abstr. 291.11). These results argue strongly against the H^+ hypothesis of lateral inhibition in its general form and suggests that another molecular mechanism

Published July 30, 2007

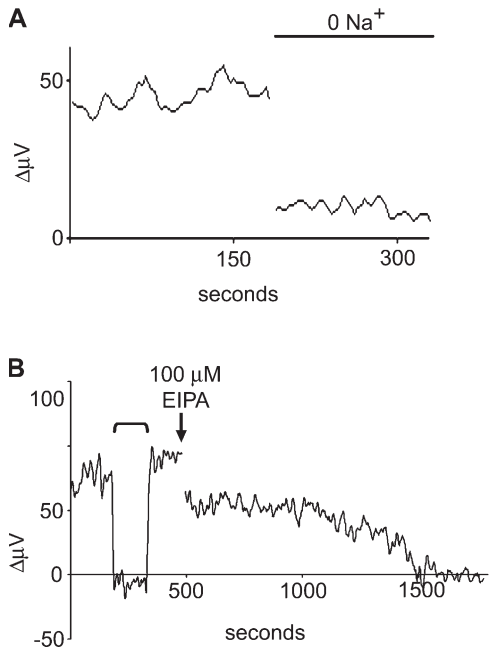

Figure 7. (A) Effects of 0 mM sodium on the standing proton flux signal from catfish cone horizontal cells. The initial portion of the trace shows the proton flux signal from one cell first in normal catfish Ringer's solution, and then when bathed in a solution in which all the sodium had been replaced by *N*-methyl-D-glucamine. (B) Effects of EIPA on the standing proton flux signal from catfish cone horizontal cells. At the arrow, 100 μM EIPA was applied to the bath; a slow decline of the H^+ signal was evident.

must be responsible for the generation of lateral inhibition within the outer retina.

Our data are consistent with the hypothesis that the increase in intracellular calcium induced by glutamate activates plasmalemma calcium Ca^{2+}/H^+ ATPase pumps that restore the resting intracellular calcium concentration by shuttling Ca^{2+} ions out of the cell in exchange for extracellular H^+ ions (Hao et al. 1994; Salvador et al. 1998). This would lead to an alkalinization of the extracellular surface, as reported here, and an intracellular acidification, as has been reported for catfish cone horizontal cells previously by Dixon et al. (1993). Support for this model comes from the observation that glutamate-induced modulation of H^+ flux is dependent on the presence of extracellular calcium and that the glutamate-induced extracellular alkalinization is significantly reduced by the PMCA inhibitor carboxyeosin. Additionally, the observation that glutamate and its analogues kainate and NMDA alkalinize the extracellular solution adjacent to the cell membrane—that is, drive the flux not just to zero, but reverse its sign—suggests the activation of a mechanism that transports H^+ ions from the extracellular milieu into the cell, rather than the simple turn-off of a H^+ extrusion mechanism. The alkalinization induced by glutamate and its analogues cannot be accounted for by simply reducing or turning off of the activity of Na^+/H^+ exchange; even complete shut down of this exchanger would reduce H^+ flux at most to zero, not to the negative values indicative of alkalinization demonstrated here.

The activation of ionotropic glutamate receptors will permit the flux of external Ca^{2+} into the cell, and consequent calcium-induced calcium release from internal stores may also play a role in the alterations in extracellular H^+ flux, as was suggested in data from previous work examining H^+ fluxes in skate horizontal cells (Molina et al., 2004). Glutamate will also permit the flux of Na^+ into the cell. It is possible to hypothesize that Na^+ entry into the cell could be large enough to reduce the Na^+ gradient, and thus potentially alter activity of a Na^+/H^+ exchanger. However, the fact that the effect of glutamate on proton flux was significantly reduced when 10 μM of the L-type calcium channel blocker nifedipine was present argues strongly against a significant involvement of Na^+/H^+ exchange in the glutamate-induced alteration of proton flux. Inhibition of the glutamate-induced response by the removal of extracellular calcium in the media also argues against a role for Na^+/H^+ exchangers. In both of these experimental conditions, glutamate should still open AMPA and NMDA channels and allow sodium into the cell. Recordings from cells under these conditions showed a persistent steady outward proton flux similar to that of control cells.

An important question concerns the magnitude of changes in H^+ concentration likely to occur under normal physiological conditions within the synaptic cavity where photoreceptors, horizontal cells, and bipolar cells contact one another. The changes in extracellular pH we have observed are small; a 100-μV signal reflects a change of ~0.002 pH units (Molina et al., 2004). However, in the experiments conducted here, hydrogen ions can readily and rapidly diffuse away into the vast sink of extracellular fluid surrounding the cells. The situation in the intact physiological system is likely to be significantly different. The invaginating synapse created by the synaptic terminals of photoreceptors tends to encapsulate the processes of horizontal cells and bipolar cells and has a very limited extracellular space (compare Mariani, 1984; Hidaka et al. 1986). This restricted and small volume is an environment in which quite small changes in the amount of H^+ ions could have a dramatic effect on the overall value of extracellular pH, acting to magnify the effects exerted by the H^+ regulatory mechanisms of horizontal cells. The numbers of protons needed to alter extracellular pH have been estimated to be quite small indeed. Based on an estimate of extracellular volume within the invaginating synaptic cleft of a photoreceptor synaptic terminal on the order of 3×10^{-18} liters (Raviola and Gilula, 1975), Vessey et al. (2005) calculated that approximately two protons would give rise to an extracellular pH of 6. These authors further estimated that the flux of protons to maintain a steady-state pH could be in the range of ~40

Published July 30, 2007

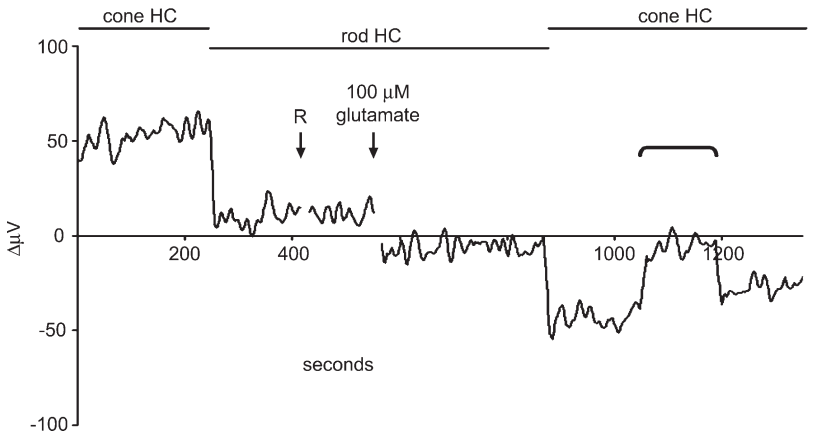

Figure 8. H^+ signals from rod and cone catfish horizontal cells. The initial portion of the trace is a self-referencing signal from a cone-driven horizontal cell. The electrode was then repositioned adjacent to a rod-driven horizontal cell (section of bar labeled "rod HC"). A differential recording was detected that was significantly smaller than that of the cone horizontal cell. At the arrow, a 1-ml bolus of catfish Ringer's solution was applied; no significant change was seen in the signal. At the second arrow, an additional 1-ml bolus of catfish Ringer's solution containing glutamate (final concentration 100 μM in the dish) was added; the signal from the rod horizontal cell was seen to now become negative. The electrode was then moved back to the cone horizontal cell (portion of the upper bar labeled "cone HC"), and a larger negative signal was detected.

protons per second per cleft. This flux represents an extremely small amount that could easily be accommodated by the action of pumps and transporters.

The self-referencing H^+-selective electrodes used here have enabled us to readily monitor changes in proton flux from single isolated cells. It is important to appreciate that the technique is unfortunately not well suited for recording changes in extracellular pH in the intact retina at the precise area where photoreceptors contact horizontal cells. The invaginating structure of the photoreceptor synapse makes it virtually impossible to place a microelectrode tip, no matter how small, in the tight, small, and highly confined area where horizontal cell, bipolar cell, and photoreceptor processes make contact. As previously noted, in work that otherwise provides support for the H^+ hypothesis of lateral inhibition, Hirasawa and Kaneko (2003) reported being unable to measure changes in extracellular pH in the outer plexiform layer as a function of surround illumination, attributing their inability to do so to this technical limitation. The inability to make such direct measurements leaves open the possibility that the mechanisms used by horizontal cells to regulate extracellular pH might be different within the synaptic specialization where photoreceptors contact the horizontal cells as compared with other parts of the horizontal cell. Novel imaging methods will need to be devised to monitor H^+ alterations in the intact synaptic cleft to address the possibility that such extracellular H^+ microdomains may exist (compare Schwiening and Willoughby, 2002; Pantazis et al., 2005). In this regard it is worth noting that our measurements were made from many different areas around hundreds of horizontal cells. In every case, we observed that glutamate induced an alkalization of the extracellular face of the cell, and never saw a single instance in which the addition of glutamate promoted an acidification around the area of the cell membrane being examined. It is also worth noting that imaging techniques such as we envision to measure the extracellular pH within the synaptic cavity still would not easily address regulation of pH by horizontal cells specifically, since the extracellular pH within the synapse would likely be a complex result of the activity of photoreceptor, bipolar, horizontal, and surrounding glial cells.

Despite the limitations mentioned above, the data we have obtained with our self-referencing H^+-selective electrodes lead us to suggest that glutamate-induced alterations in H^+ flux from horizontal cells act normally in a manner precisely opposite from that of the original H^+ hypothesis. Rather than inducing surround inhibition, we hypothesize that horizontal cell–mediated reduction in extracellular H^+ may lead to a resensitization of the cone synapse. DeVries (2001) reported that the fusion of photoreceptor vesicles with the plasma membrane is sufficient to induce a temporary inhibition of neurotransmitter release of mammalian photoreceptors, due to blockade of photoreceptor calcium channels by the H^+ ions released in the process of synaptic transmission. Hosoi et al. (2005) similarly found that protons exocytosed from cone photoreceptors of the newt retina could inhibit calcium currents of cone (but not rod) photoreceptors in the newt retina. We believe that glutamate released by the photoreceptors depolarizes the horizontal cells, inducing calcium influx and consequent activation of the horizontal cell PMCA pumps, leading to a reduction of the proton concentration in the extracellular space. This would promote additional release of glutamate by relieving the proton block on photoreceptor calcium channels, permitting the opening of photoreceptor calcium channels. It is also possible that the alkalinization induced by glutamate could potentially relieve H^+ block of postsynatpic glutamate receptors (Wu and Christensen, 1996). In both cases, the effects of glutamate on postsynaptic elements would be augmented. Thus, the glutamate-induced alteration in H^+ flux from horizontal cells has the potential to act as a mechanism to enhance the effects of glutamate within the outer plexiform layer of the retina.

Indeed, we suggest that this may be a common feature of excitatory synapses in the retina in particular and the nervous system in general—that presynaptic release of neurotransmitter is accompanied by acidification of the synapse, and that post-synaptic elements may play a role in reducing that acidification, thus relieving H^+ block of calcium channels and resensitizing the synapses for further release of neurotransmitter.

We thank Richard Sanger for expert computer and hardware assistance and Craig Hamilton for preparation of silanized pipettes used to construct H^+-selective electrodes. We are also grateful to Steve Kahrs at Osage Catfisheries, Inc., for kindly arranging custom shipments of excellent quality catfish at all times of the year.

This work was supported by an MBL Summer Research Fellowship (the Herbert W. Rand Fellowship, the Lucy B. Lemann Fellowship, and the Erik B. Fries Endowed Fellowship) (M.A. Kreitzer), a Lilly Scholarship Initiative Award\Indiana Wesleyan University (M.A. Kreitzer), grant 009-1281 from the National Science Foundation (R.P. Malchow), grant P41 RR001395 from the National Center for Research Resources (P.J.S. Smith), and the Independent Research and Development Program of the National Science Foundation. The findings, opinions, and conclusions expressed in this publication are those of the authors and do not necessarily reflect the views of the National Science Foundation.

Olaf S. Andersen served as editor.

Submitted: 5 January 2007
Accepted: 5 July 2007

REFERENCES

Barnes, S., V. Merchant, and F. Mahmud. 1993. Modulation of transmission gain by protons at the photoreceptor output synapse. *Proc. Natl. Acad. Sci. USA.* 90:10081–10085.

Baylor, D.A., M.G. Fuortes, and P.M. O'Bryan. 1971. Receptive fields of cones in the retina of the turtle. *J. Physiol.* 214:265–294.

Cadetti, L., and W.B. Thoreson. 2006. Feedback effects of horizontal cell membrane potential on cone calcium currents studied with simultaneous recordings. *J. Neurophysiol.* 95:1992–1995.

Caramelo, O.L., P.F. Santos, A.P. Carvalho, and C.B. Duarte. 1999. Metabotropic glutamate receptors modulate [(3)H]acetylcholine release from cultured amacrine-like neurons. *J. Neurosci. Res.* 58:505–514.

Cevc, G. 1990. Membrane electrostatics. *Biochim. Biophys. Acta.* 1031:311–382.

Chesler, M. 1990. The regulation and modulation of pH in the nervous system. *Prog. Neurobiol.* 34:401–427.

Chesler, M. 2003. Regulation and modulation of pH in the brain. *Physiol. Rev.* 83:1183–1221.

Choi, H.S., and D.A. Eisner. 1999. The role of sarcolemmal Ca^{2+}-ATPase in the regulation of resting calcium concentration in rat ventricular myocytes. *J. Physiol.* 515:109–118.

Coleman, P.A., and R.A. Miller. 1988. Do N-methyl-D-aspartate receptors mediate synaptic responses in the mudpuppy retina? *J. Neurosci.* 8:4728–4733.

Copenhagen, D.R., and C.E. Jahr. 1989. Release of endogenous excitatory amino acids from turtle photoreceptors. *Nature.* 341:536–539.

Davis, S.F., and C.L. Linn. 2003. Mechanism linking NMDA receptor activation to modulation of voltage-gated sodium current in distal retina. *Am. J. Physiol. Cell Physiol.* 284:C1193–C1204.

DeVries, S.H. 2001. Exocytosed protons feedback to suppress the Ca^{2+} current in mammalian cone photoreceptors. *Neuron.* 32:1107–1117.

DeVries, S.H., and E.A. Schwartz. 1989. Modulation of an electrical synapse between solitary pairs of catfish horizontal cells by dopamine and second messengers. *J. Physiol.* 414:351–375.

Deitmer, J.W., and C.R. Rose. 1996. pH regulation and proton signaling by glial cells. *Prog. Neurobiol.* 48:73–103.

Dixon, D.B., K. Takahashi, and D.R. Copenhagen. 1993. L-glutamate suppresses calcium current in catfish horizontal cells by raising intracellular proton concentration. *Neuron.* 11:267–277.

Dong, C.J., S.A. Picaud, and F.S. Werblin. 1994. GABA transporters and GABAC-like receptors on catfish cone-but not rod-driven horizontal cells. *J. Neurosci.* 14:2648–2658.

Fierro, L., R. DiPolo, and I. Llano. 1998. Intracellular calcium clearance in Purkinje cell somata from rat cerebellar slices. *J. Physiol.* 510:499–512.

Fluka. 1996. Selectophore, Ionophores for Ion-selective Electrodes and Optrodes. Fluka Chemie A.G., Ronkonkoma, New York. 153 pp.

Gafka, A.C., and C.L. Linn. 2001. Modulation of a voltage-gated calcium channel linked to activation of glutamate receptors and calcium-induced calcium release in the catfish retina. *J. Physiol.* 535:47–63.

Gafka, A.C., K.S. Vogel, and C.L. Linn. 1999. Evidence of metabotropic glutamate receptor subtypes found on catfish horizontal and bipolar retinal neurons. *Neuroscience.* 90:1403–1414.

Hanitzsch, R., and L. Kuppers. 2001. The influence of HEPES on light responses of rabbit horizontal cells. *Vision Res.* 41:2165–2172.

Hao, L., J.L. Rigaud, and G. Inesi. 1994. Ca^{2+}/H^+ countertransport and electrogenicity in proteoliposomes containing erythrocyte plasma membrane Ca-ATPase and exogenous lipids. *J. Biol. Chem.* 269:14268–14275.

Hare, W.A., and W.G. Owen. 1998. Effects of bicarbonate versus HEPES buffering on measured properties of neurons in the salamander retina. *Vis. Neurosci.* 15:263–271.

Harsanyi, K., and S.C. Mangel. 1993. Modulation of cone to horizontal cell transmission by calcium and pH in the fish retina. *Vis. Neurosci.* 10:81–91.

Hille, B. 1992. Modifiers of gating. *In* Ionic Channels of Excitable Membranes. Sinauer Associates Inc., Sunderland, MA. 445–471.

Hidaka, S., B.N. Christensen, and K. Naka. 1986. The synaptic ultrastructure in the outer plexiform layer of the catfish retina: a three-dimensional study with HVEM and conventional EM of Golgi-impregnated bipolar and horizontal cells. *J. Comp. Neurol.* 247:181–199.

Hirasawa, H., and A. Kaneko. 2003. pH changes in the invaginating synaptic cleft mediate feedback from horizontal cells to cone photoreceptors by modulating Ca^{2+} currents. *J. Gen. Physiol.* 122:657–671.

Hosoi, N., I. Arai, and M. Tachibana. 2005. Group III metabotropic glutamate receptors and exocytosed protons inhibit L-type calcium currents in cones but not in rods. *J. Neurosci.* 25:4062–4072.

Kleinschmidt, J. 1991. Signal transmission at the photoreceptor synapse. Role of calcium ions and protons. *Ann. NY Acad. Sci.* 635:468–470.

Kolb, H., R. Nelson, P. Ahnelt, and N. Cuenca. 2001. Cellular organization of the vertebrate retina. *Prog. Brain Res.* 131:3–26.

Kreitzer, M.A., K.A. Andersen, and R.P. Malchow. 2003. Glutamate modulation of GABA transport in retinal horizontal cells of the skate. *J. Physiol.* 546:717–731.

Kuhtreiber, W.M., and L.F. Jaffe. 1990. Detection of extracellular calcium gradients with a calcium-specific vibrating electrode. *J. Cell Biol.* 110:1565–1573.

Linn, C.P., and B.N. Christensen. 1992. Excitatory amino acid regulation of intracellular Ca^{2+} in isolated catfish cone horizontal cells measured under voltage- and concentration-clamp conditions. *J. Neurosci.* 12:2156–2164.

Published July 30, 2007

Mariani, A.P. 1984. The neuronal organization of the outer plexiform layer of the primate retina. *Int. Rev. Cytol.* 86:285–320.

McLaughlin, S., N. Mulrine, T. Gresalfi, G. Vaio, and A. McLaughlin. 1981. Adsorption of divalent cations to bilayer membranes containing phosphatidylserine. *J. Gen. Physiol.* 77:445–473.

McLaughlin, S.G., G. Szabo, and G. Eisenman. 1971. Divalent ions and the surface potential of charged phospholipid membranes. *J. Gen. Physiol.* 58:667–687.

Molina, A.J.A., M.P. Verzi, A.D. Birnbaum, M.A. Kreitzer, E.N. Yamoah, K. Hammar, P.J.S. Smith, and R.P. Malchow. 2004. Neurotransmitter modulation of extracellular pH fluxes from retinal horizontal cells. *J. Physiol.* 560:639–657.

Nguyen, H.V., G.E. Shull, and J.E. Melvin. 2000. Muscarinic receptor-induced acidification in sublingual mucous acinar cells: loss of pH recovery in Na^+-H^+ exchanger-1 deficient mice. *J. Physiol.* 523:139–146.

Oakley, B., II, and R. Wen. 1989. Extracellular pH in the isolated retina of the toad in darkness and during illumination. *J. Physiol.* 419:353–378.

O'Dell, T.J. 1989. Pharmacological characterization of voltage-clamped catfish rod horizontal cell responses to kainic acid. *Brain Res.* 477:118–125.

O'Dell, T.J., and B.N. Christensen. 1989. Horizontal cells isolated from catfish retina contain two types of excitatory amino acid receptors. *J. Neurophysiol.* 61:1097–1109.

Pande, J., K.K. Mahli, A. Sawh, M.M. Szewczyk, F. Simpson, and A.K. Grover. 2006. Aortic smooth muscle and endothelial plasma membrane Ca^{2+}-pump isoforms are inhibited differentially by the extracellular inhibitor caloxin 1b1. *Am. J. Physiol. Cell Physiol.* 290:C1341–C1349.

Pantazis, A., P. Keegan, M. Postma, and C.J. Schwiening. 2005. The effect of neuronal morphology and membrane-permeant weak acid and base on the dissipation of depolarization-induced pH gradients in snail neurons. *Pflugers Arch.* 452:175–187.

Raviola, E., and N.B. Gilula. 1975. Intramembrane organization of specialized contacts in the outer plexiform layer of the retina. *J. Cell Biol.* 65:192–222.

Salvador, J.M., G. Inesi, J.L. Rigaud, and A.M. Mata. 1998. Ca^{2+} transport by reconstituted synaptosomal ATPase is associated with H^+ countertransport and net charge displacement. *J. Biol. Chem.* 273:18230–18234.

Schwiening, C., H.J. Kennedy, and R.C. Thomas. 1993. Calcium-hydrogen exchange by the plasma membrane Ca-ATPase of voltage-clamped snail neurons. *Proc. R. Soc. Lond. B. Biol. Sci.* 253:285–289.

Schwiening, C.J., and D. Willoughby. 2002. Depolarization-induced pH microdomains and their relationship to calcium transients in isolated snail neurones. *J. Physiol.* 538:371–382.

Shingai, R., and B.N. Christensen. 1986. Excitable properties and voltage-sensitive ion conductances of horizontal cells isolated from catfish (*Ictalurus punctatus*) retina. *J. Neurophysiol.* 56:32–49.

Smith, P.J., and J. Trimarchi. 2001. Noninvasive measurement of hydrogen and potassium ion flux from single cells and epithelial structures. *Am. J. Physiol. Cell Physiol.* 280:C1–C11.

Smith, P.J., K. Hammar, D.M. Porterfield, R.H. Sanger, and J.R. Trimarchi. 1999. Self-referencing, non-invasive, ion selective electrode for single cell detection of trans-plasma membrane calcium flux. *Microsc. Res. Tech.* 46:398–417.

Smith, P.J.S., R.S. Sanger, and M.A. Messerli. 2007. Principles, development and applications of self-referencing electrochemical microelectrodes to the determination of fluxes at cell membranes. *In* Methods and New Frontiers in Neuroscience. Adrian C. Michael, editor. CRC Press, Boca Raton, FL. 373–405.

Somieski, P., and W. Nagel. 2001. Measurement of pH gradients using an ion-sensitive vibrating probe technique (IP). *Pflugers Arch.* 442:142–149.

Szamier, R.B., and H. Ripps. 1983. The visual cells of the skate retina: structure, histochemistry, and disc-shedding properties. *J. Comp. Neurol.* 215:51–62.

Vessey, J.P., A.K. Stratis, B.A. Daniels, N. Da Silva, M.G. Jonz, M.R. Lalonde, W.H. Baldridge, and S. Barnes. 2005. Proton-mediated feedback inhibition of presynaptic calcium channels at the cone photoreceptor synapse. *J. Neurosci.* 25:4108–4117.

Wanaverbecq, N., S.J. Marsh, M. Al-Qatari, and D.A. Brown. 2003. The plasma membrane calcium-ATPase as a major mechanism for intracellular calcium regulation in neurones from the rat superior cervical ganglion. *J. Physiol.* 550:83–101.

Werblin, F.S., and J.E. Dowling. 1969. Organization of the retina of the mudpuppy, Necturus maculosus. II. Intracellular recording. *J. Neurophysiol.* 32:339–355.

Wu, S.M. 1992. Feedback connections and operation of the outer plexiform layer of the retina. *Curr. Opin. Neurobiol.* 2:462–468.

Wu, X., and B.N. Christensen. 1996. Proton inhibition of the NMDA-gated channel in isolated catfish cone horizontal cells. *Vision Res.* 36:1521–1528.