Intracellular pH modulates inner segment calcium homeostasis in vertebrate photoreceptors

David Križaj,1,2 Aaron J. Mercer,3 Wallace B. Thoreson,3,4 and Peter Barabas1

1Departments of Ophthalmology and Visual Sciences, Moran Eye Center, and 2Physiology, University of Utah School of Medicine, Salt Lake City, Utah; and 3Departments of Ophthalmology and Visual Sciences and 4Pharmacology and Experimental Neurosciences, University of Nebraska Medical Center, Omaha, Nebraska

Submitted 6 July 2010; accepted in final form 28 September 2010

Križaj D, Mercer AJ, Thoreson WB, Barabas P. Intracellular pH modulates inner segment calcium homeostasis in vertebrate photoreceptors. Am J Physiol Cell Physiol 300: C187–C197, 2011. First published September 29, 2010; doi:10.1152/ajpcell.00264.2010.—Neuronal metabolic and electrical activity is associated with shifts in intracellular pH (pHi) proton activity and state-dependent changes in activation of signaling pathways in the plasma membrane, cytosol, and intracellular compartments. We investigated interactions between two intracellular messenger ions, protons and calcium (Ca2+), in salamander photoreceptor inner segments loaded with Ca2+ and pH indicator dyes. Resting cytosolic pH in rods and cones in HEPES-based saline was acidified by ~0.4 pH units with respect to pH of the superfusing saline (pH = 7.6), indicating that dissociated inner segments experience continuous acid loading. Cytosolic alkalinization with ammonia chloride (NH4Cl) depolarized photoreceptors and stimulated Ca2+ release from internal stores, yet paradoxically also evoked dose-dependent, reversible decreases in [Ca2+]i. Alkalization-evoked [Ca2+]i decreases were independent of voltage-operated and store-operated Ca2+ entry, plasma membrane Ca2+ extrusion, and Ca2+ sequestration into internal stores. The [Ca2+]i-suppressive effects of alkalinization were antagonized by the fast Ca2+ buffer BAPTA, suggesting that pHi directly regulates Ca2+ binding to internal anionic sites. In summary, this data suggest that endogenously produced protons continually modulate the membrane potential, release from Ca2+ stores, and intracellular Ca2+ buffering in rod and cone inner segments.

Activity-dependent pHi and [Ca2+]i changes are especially pronounced in metabolically active cells, such as vertebrate photoreceptors (26, 29, 38, 59, 80). Light and darkness are associated with intracellular production and export of protons (11, 21, 26, 37–38). Addition of approximately two protons to the restricted extracellular volume surrounding rods and cones was calculated to acidify the extracellular pH from 7.4 to ~6. Continuous acid loading can have marked consequences for photoreceptor light sensitivity and output (31–32, 45, 77). Accordingly, experimental manipulation of extracellular pH (pHo) affects virtually every level of Ca2+-dependent process in rods and cones, including membrane surface charge (4), operating range of voltage-gated channels in the inner segment region (3, 5, 21, 26, 77), activity of acid-sensing ion channels (22), cGMP-dependent light-regulated channels in the outer segment (38, 45), ERG a-wave (38), synaptic transmission (5, 37), and synaptic feedback (23, 31, 77). While pronounced, these effects of pHo do not provide straightforward insights into the signaling function of intracellularly generated protons. Despite the known susceptibility of intracellular Ca2+ signals to endogenous protons, little is known about proton-Ca2+ interactions in photoreceptor cytosol. Moreover, recent studies have implicated changes in photoreceptor pHi in dysfunctional Ca2+ homeostasis and photoreceptor degeneration (10, 83), further underscoring the need to understand pHi-[Ca2+]i interactions in the inner segment where the apoptotic machinery is localized.

The purpose of this paper is to characterize the effects of pH modulation on Ca2+ homeostasis in rods and cones. pH in physiological experiments on dissociated retinal cells is typically buffered with HEPES, which has been the buffer of choice used in investigations of voltage-operated and store-operated Ca2+ entry (SOCE) and Ca2+ release from internal stores in retinal photoreceptors, horizontal cells, bipolar cells, glial cells, and central neurons (3–5, 39–41, 46, 48, 50, 55, 60, 63–71, 73, 79). In this study, we systematically characterized the effects of changing pHi on voltage-operated Ca2+ channels, Ca2+ stores, SOCE, cytosolic protein buffers, and buffering power of the photoreceptor cytosol. We used the ammonium prepulse method that has no effect on pHo and therefore mimics the effects of metabolically produced protons (12–13, 67). Our results suggest that intracellular pH represents a comprehensive signaling mechanism that simultaneously modulates Ca2+ entry, extrusion, sequestration/release from intracellular stores, and the availability of Ca2+-binding sites in the inner segment cytosol.

MATERIALS AND METHODS

Isolation of cells. All procedures were approved by institutional animal care and use committees at the University of Utah and...
University of Nebraska Medical Center following the guidelines established by Association for Research in Vision and Ophthalmology and The Society for Neuroscience. Aquatic-stage tiger salamanders (Ambystoma tigrinum) were purchased from Charles Sullivan (Nashville, TN) and kept in filtered water at 4°C and 12:12-h light-dark cycle. After decapitation and pithing, retinas were dissected from enucleated eyes, and photoreceptors were isolated using either papain (7 U/ml) (to obtain intact cells) or mechanical isolation (to obtain rod outer segments). After being washed, cells were triturated in L-15 medium, plated onto sterilized clean glass coverslips coated with concanavalin A (1 mg/ml; Sigma-Aldrich), and allowed to settle for 40 min. Dissociated rod and cone inner segments were unambiguously identified by the morphology of the respective cell bodies and ellipsoids (e.g., 39, 48, 55, 64). Most extracellular solutions were HCO₃⁻/CO₂-free to minimize activation of HCO₃⁻/Cl⁻ exchange mechanisms and contained (in mM): 97 NaCl, 2 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, 20 glucose, 1 pyruvic acid, 2 lactic acid, and 0.3 ascorbic acid. 

Intracellular buffering capacity of lower vertebrate cells (resulting in pHo of 7.4 at 240 mOsm. The 10 mM HEPES has a minimal effect on the pH). After decapitation and pithing, retinas were dissected from enucleated eyes, and photoreceptors were isolated using either papain (7 U/ml; Sigma-Aldrich), and allowed to settle for 20 min. In previous studies, we have shown that dissociated rod and cone inner segments loaded with Ca²⁺ indicator dye dyes remain healthy for several hours without adverse effects on baseline [Ca²⁺]i or the amplitude of depolarization-evoked [Ca²⁺]i responses (39–45, 63–66, 68–69). Fluorescence imaging was performed on an inverted Nikon Ti or an upright 600EF microscope using cooled 14-bit interline charge-coupled device cameras with 1,392 × 1,040 pixel imaging arrays and 6.45 × 6.45 μm pixel size (Coolsnap HQ2; Photometrics, Tucson, AZ) and ×40 (1.3 numerical aperture oil and 0.8 numerical aperture water) objectives. Cameras were cooled at −40°C with the readout set at 10 MHz. Excitation was delivered through the epi-port via a 5-mm liquid light guide (300 series; Sutter Instruments, Novato, CA) attached to a 150 W Xenon arc lamp equipped with a Smart Shutter (Sutter Instruments). Band-pass filters (located fura-2 and BCFCE filter sets; Chromo or Omega, Brattleboro, VT) were positioned serially in the pathway to provide excitation illumination; appropriate emission filters were positioned within dichroic mirror cubes. Single wavelength 490-nm excitation using a band-pass dichroic mirror in the BCFCE filter set was used for Fura-4 excitation. Circular regions of interest encompassed the central portion of cell body and ellipsoid regions; unless otherwise stated, only the cell body data are shown. Image acquisition was run in the continuous mode at 0.2–0.4 Hz and binned at 3 × 3. In a subset of cells, [Ca²⁺]i and pH were recorded from fura-2 AM and BCFCE-AM loaded cells by using a dual fura-2/BCFCE filter (Chroma set version 72000). In these experiments, a 340–to-380 ratio was acquired 2 s following acquisition of each 495-to-440 ratio. Background fluorescence was measured in identical regions of interests in neighboring areas on the coverslip devoid of cells. After sequential image acquisition of cells’ fluorescence at 495/440 nm or 340/380 nm, the backgrounds were subtracted using commercial software (Metafluor; Universal Imaging, West Chester, PA and NIS Elements, Melville, NY). Calibration of [Ca²⁺]i, and pH was carried out as described previously (63–66). Intracellular pH was calibrated from 495-to-440 ratios using the nigericin/high extracellular K⁺ method (10 μM nigericin/90 mM KC1). External KC1 in the calibration solution was raised to 90 mM to minimize the K⁺ gradient across the plasma membrane. A separate line was used to deliver pH calibration solutions, and the tubing was washed with a prolonged (>1 h) ethanol wash after each experiment (8, 57, 67).

Mn²⁺ quenching showed that 95% of the fluorescence signal originated from the cytosol (66). To block plasma membrane Ca²⁺ extrusion and store-operated channels, mM concentrations of extracellular La³⁺ ([La³⁺]) were used in some experiments. La³⁺ had little effect on intensity of fluorescence emission at fura-2 excitation wavelengths. Fura-2 fluorescence ratios were calibrated as previously reported with 10 μM ionomycin and 10 mM Ca²⁺ (Rmax) or 0 Ca²⁺/3 mM EGTA (Rmin), assuming a dissociation constant for Ca²⁺/fura 2 at room temperature of 224 nM. The association and dissociation rate constants for fura-2 are insensitive to pH over the range of 7.4 to 8.4 (54, 85). However, the Keq of fura-2 is decreased as pH falls below 6.5 (44, 59, 54). As pH was, typically above 7.0 in this study, Keq corrections would have little impact on the final value of [Ca²⁺]i, and were therefore not performed.

Fluorescence data from experiments using the single-wavelength indicator Furo-4 were normalized as ΔF/F. For uncaging experiments, cells were incubated simultaneously with 20 μM of nitrophenyl (NP)-EGTA AM and 5 μM Fluo-4 AM (Invitrogen) for 20 min; Ca²⁺ was uncaged by 5-ms flashes of white light (340–700 nm broadband excitation at 150 W, Lambda DG4; Sutter Instruments) delivered via the liquid light guide from the Xenon arc lamp. Periodic 490-nm excitation of Fluo-4 was briefly paused during NP-EGTA uncaging using a custom-designed macro program in Metafluor. After delivery of the uncaging flash, the acquisition of Fluo-4 emission data immediately resumed.

Electrophysiology. For recording and imaging experiments, isolated photoreceptors were superfused with an extracellular medium bubbled with 100% O₂ containing (in mM): 116 NaCl, 2.5 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 5 glucose, and 10 HEPES (pH 7.8). Intracellular [Ca²⁺]i increases were evoked by bath application of a solution containing 20 mM KC1. As shown previously (39–41, 55, 63–66, 68–69), high K⁺-evoked depolarization elevates inner segment [Ca²⁺]i due to the combined effect of activated L-type channels and release from Ca²⁺ stores. Depolarization evokes a transient [Ca²⁺]i peak, whereupon [Ca²⁺]i relaxes to steady-state levels due to the combined effect of L-type channel inactivation and depletion of Ca²⁺ stores (55, 66). The effects of pH on [Ca²⁺]i were always examined after the steady-state [Ca²⁺]i plateau was achieved. The high K⁺ solution was prepared by equimolar replacement of KC1 for NaCl. NH₄Cl (3, 10, and 15 mM) was added by dilution from a 1 M stock solution. Cells were loaded with Fura 2-AM as described above. We used a gramicidin-perforated patch technique to simultaneously measure native membrane potentials in rods and cones without disturbing Fura 2-loading. Electodes were pulled using a PP-830 vertical puller (Narishige, East Meadow, NY) from borosilicate glass pipettes (1.2 mm OD, 0.9 mm ID, with an internal filament, World Precision Instruments, Sarasota, FL) with tips 2–3 μm in diameter and resistance values between 12 and 18 MΩ. The patch pipette solution contained (in mM): 40 KC1, 55 KCH3SO3, 3.5 NaCl, 2 glucose, 1 CaCl2, 1 MgCl2, 10 Mg ATP, 0.5 GTP, 5 EGTA, 1 HEPES, 1 reduced glutathione, and 5 μg/ml gramicidin (pH 7.2). Isolated photoreceptors were voltage clamped using a Multiclamp amplifier, Digidata 1322 interface, and pClamp 9.2 (MDS Analytical Devices, Sunnyvale, CA). After establishing a high-resistance seal, we waited ~2–3 min to allow gramicidin to perfuse the plasma membrane before measuring membrane potential in current clamp recording mode. Membrane potentials were recorded at a sampling rate of 500 ms/point.

RESULTS

The resting membrane potential of photoreceptors in darkness is further hyperpolarized in the light due to closure of light-sensitive cGMP-dependent channels in outer segments of rods and cones. Because the majority of enzymatically dissociated photoreceptors lost their outer segments, we could not use light to set the adaptation state of the cells. Instead, we used high and low concentrations of potassium to depolarize or hyperpolarize rod and cone inner segments loaded with fura-2 or Fluor-4 indicator dyes. The 20 mM KCl depolarized diso-
Ammonium chloride modulates pH_i in inner segments of rods and cones. pH_i within inner segment cytosol was modulated by using the ammonium prepulse technique (12–13, 67). Dissolution of ammonium chloride (NH_4Cl) in the external saline produces the ammonia gas (NH_3), which readily crosses the plasma membrane into the cell interior where it ionizes into NH_4^+. By binding cytosolic protons, ammonia alkalinizes the cell interior. Removal of NH_4Cl from the bathing solution reverses the above sequence, producing a rebound acidification as NH_3 diffuses from the cytosol and leaves residual protons behind (11). The ammonium pulse triggered an alkalinization in photoreceptor inner segments (Fig. 1), which was followed by rebound acidification upon washout (seen as upward and downward deflections, respectively, in pH_i traces of Figs. 1B and S1) (Supplemental figures for this article are available online at the *American Journal of Physiology–Cell Physiology* website.). Average pH_i levels in dissociated inner segments, measured with the proton indicator dye BCECF, were 7.15 ± 0.02 (mean ± SE; n = 9) in rods and 7.32 ± 0.04 (n = 13) in cones. For both rods and cones, 15 mM NH_4Cl increased pH_i from 7.20 ± 0.02 to 7.60 ± 0.02 units from the physiological pH_i of 7.2 to pH_i 7.6 (i.e., the pH decrease in 63 to ~10 nM. An acid shift of 0.22 ± 0.02 was observed after NH_4Cl washout with control saline.

**Intracellular protons modulate [Ca^{2+}]_i in depolarized cells.** The effect of pH_i on [Ca^{2+}]_i was studied in cells depolarized with high KCl to [Ca^{2+}]_i plateaus that roughly approximated in vivo levels under dark adapted conditions. The amplitudes of NH_4Cl-evoked pH_i changes in depolarized photoreceptors (20 mM KCl) stimulated with 15 mM NH_4Cl were 96.1 ± 2.5% (cones, n = 5; P = 0.1999, paired t-test) and 99.0 ± 4.9% (rods, n = 3; P = 0.8585, paired t-test) of responses observed under control conditions (2 mM external KCl) (Fig. 1, B and C).

The effectiveness of changes in pH_i on inner segment [Ca^{2+}]_i, was a function of steady-state [Ca^{2+}]_i levels. Alkalization of hyperpolarized cells at low baseline [Ca^{2+}]_i, evoked small (<50 nM) transient [Ca^{2+}]_i increases above baseline [Ca^{2+}]_i in 19/35 cells; no consistent effect on [Ca^{2+}]_i, baseline was detected in 15 of 36 photoreceptors, and a decrease occurred in two cells. Because these effects were small and variable, they were not studied further. In contrast, the ammonium prepulse consistently reduced [Ca^{2+}]_i levels in depolarized photoreceptors. As shown in Figs. 2 and S1, alkalinization in the presence of 20 mM KCl elicited sustained [Ca^{2+}]_i decreases, whereas the [Ca^{2+}]_i rebound during NH_4Cl washout with control high-K^+ saline coincided with the acidification phase. The change in pH_i does not have an artefactual effect on the dye itself, as seen by the opposing shift in fluorescence emission evoked by 340 and 380 nm excitations in alkalinized cells (Fig. S1, bottom). Amplitudes of alkalinization-induced [Ca^{2+}]_i decreases were comparable in rods and cones. In eight depolarized rods, 15 mM NH_4Cl reversibly decreased [Ca^{2+}]_i, measured at the plateau levels from 258 ± 34 nM to 163 ± 24 nM (P < 0.001; paired t-test); in cones, [Ca^{2+}]_i, was decreased by 142 ± 18 nM from the plateau of 263 ± 31 nM (n = 14) (P < 0.001; paired t-test). Similar NH_4Cl-dependent effects on 340-to-380 nm ratios were observed in >200 uncalibrated photoreceptor cells.

The amplitude of NH_4Cl-evoked reductions in [Ca^{2+}]_i, was a function of NH_4Cl concentration (Figs. 2, C and E). pH_i signals were measured in BCECF-loaded cells stimulated with 0.3–15 mM NH_4Cl. In a separate set of experiments, [Ca^{2+}]_i responses to the same concentrations of NH_4Cl were measured in cells loaded with fura-2. A plot of [Ca^{2+}]_i versus pH_i at each NH_4Cl concentration revealed an inverse linear relationship between the two variables (Fig. 2F). Thus, alkalinizing cells by 0.4 pH units from the physiological pH_i of 7.2 to pH_i 7.6 (i.e., the pH of the superfusing saline) caused ~90 nM decrease in [Ca^{2+}]_i.

We independently tested the hypothesis that pH_i regulates [Ca^{2+}]_i by taking advantage of the ability of the K^+/H^+ ionophore nigericin to equilibrate the H^+ gradient across the plasma membrane. KCl concentration in these experiments was raised to 90 mM to minimize the transmembrane K^+ gradient. Fig. 2F shows the [Ca^{2+}]_i response to 10 μM nigericin at pH_o = 7.6. Since inner segment cytosol is acidified compared with the superfusing saline (Fig. 1), exposure to nigericin was equivalent to intracellular alkalinization by ~0.4 pH units. Nigericin evoked [Ca^{2+}]_i decreases in 8/11
cells (5 cones and 6 rods; $P = 0.0207$, Kruskall-Wallis nonparametric ANOVA), consistent with the idea that alkalinization decreases $[\text{Ca}^{2+}]_i$.

The effect of NH$_4$Cl on $[\text{Ca}^{2+}]_i$ in depolarized inner segments is illustrated graphically for a cone in Fig. 3. The initial fura-2 ratio in this cell was low throughout the perikaryal and ellipsoid regions. Depolarization with 20 mM KCl increased $[\text{Ca}^{2+}]_i$ in the cell body but induced a smaller $[\text{Ca}^{2+}]_i$ elevation in the mitochondria-rich ellipsoid region (arrowhead). The 10 mM NH$_4$Cl elicited a global $[\text{Ca}^{2+}]_i$ decrease across all inner...
NH4Cl-evoked decrease in [Ca2+]i, is not modulated by plasma membrane Ca2+ entry and extrusion. A decrease in [H+]i, in the photoreceptor inner segment could reduce [Ca2+]i, via a number of different but not mutually exclusive mechanisms, including: 1) hyperpolarization of the membrane potential, which would lead to decreased Ca2+ influx through plasma membrane Ca2+ channels; 2) increased sequestration into intracellular compartments; 3) increased extrusion by plasma membrane Ca2+-ATPases (PMCA); 4) modulation of store-operated channels and/or 5) changes in the equilibrium at Ca2+-H+ binding sites of intracellular buffer proteins. We investigated each of these possibilities.

Isolated rod and cone photoreceptors were examined under perforated patch conditions to compare the effects of NH4Cl-induced changes to membrane potential and [Ca2+]i. Fig. 4, A and B shows recordings of membrane potential and [Ca2+]i, (340-to-380 ratio of fura-2 fluorescence) measured simultaneously in a rod photoreceptor. In this example, 20 mM K+ depolarized the rod from a resting potential of −40 mV to −28 mV. Subsequent application of increasing concentrations of NH4Cl further depolarized the cell to a maximum of −6 mV (Fig. 4A).

Although one would normally expect depolarization to increase [Ca2+]i, extracellular NH4Cl had the opposite effect: [Ca2+]i levels in the soma decreased by up to 66% in the presence of 15 mM NH4Cl. The 15 mM NH4Cl depolarized rods by an average of −11.08 ± 2.7 mV (Fig. 4C, P < 0.0001, n = 6) but decreased the 340-to-380 ratio by 71.8% (Fig. 4D, P < 0.0001, n = 3). We also observed similarly significant depolarizing changes in the membrane potential of cone photoreceptors in response to NH4Cl application (P < 0.0001, n = 7, data not shown). These data indicate that alkalinization does not suppress Ca2+ influx by reducing the voltage-dependent activation of Ca2+ channels in the inner segment.

Alkalization-evoked [Ca2+]i decrease is not caused by modulation of Ca2+ release from internal stores or store-operated Ca2+ entry. Although the large majority of depolarized photoreceptors responded to alkalinization with a sustained decrease in [Ca2+]i, transient dose-dependent [Ca2+]i increases were observed in a subset of depolarized photoreceptors (117 ± 25 nM; n = 11; arrowheads in Fig. 5, A and B).

Alkalization-induced [Ca2+]i elevations were only observed in rods, whereas cone inner segments always displayed sustained [Ca2+]i decreases in response to NH4Cl. Transient [Ca2+]i increases in rod inner segments were antagonized by the SERCA blocker cyclopiazonic acid (CPA; 5 μM), suggesting that alkalization modulates Ca2+ release from intracellular stores. Neither CPA (Fig. 5B; n = 15) nor thapsigargin (1 μM; n = 4, data not shown) antagonized [Ca2+]i decreases evoked by the ammonium prepulse, indicating that sequestra-
tion into endoplasmic reticulum (ER) stores is not responsible for the generation of alkalinization-evoked [Ca\(^{2+}\)] increases. We next tested whether alkalinization regulates SOCE, a plasma membrane influx pathway that provides a significant fraction of Ca\(^{2+}\) entry in light-adapted inner segments (65–66). The standard overshoot technique was employed to characterize SOCE (9, 65). Ca\(^{2+}\) stores were depleted by 10-min exposure to Ca\(^{2+}\)-free saline supplemented with 5 mM CPA. Store depletion activates SOCE, which manifests in the form of transient [Ca\(^{2+}\)] elevations that follow the return from Ca\(^{2+}\)-free to Ca\(^{2+}\)-containing saline (arrows in Fig. 5C). Alkalinization evoked no change in 12 of 16 rods (75%) and 14 of 28 cones (50%). SOCE was decreased by alkalinization in four rods and nine cones, whereas an increase was observed in five cones. The average change of SOCE amplitude during exposure to NH\(_4\)Cl was 3.0 ± 6.8% (Fig. 5D), suggesting that pH\(_i\) is not a major regulator of photoreceptor SOCE.

The data summarized in Figs. 1–5 show that pH\(_i\) profoundly modulates [Ca\(^{2+}\)] in depolarized photoreceptors. pH\(_i\) modulates the inner segment membrane potential and release from internal stores but has little effect on SOCE. Its main effect, sustained decrease in [Ca\(^{2+}\)] evoked by intracellular alkalinization, is, however, independent of the membrane potential, voltage-operated Ca\(^{2+}\) entry, SOCE, and Ca\(^{2+}\) stores.

pH\(_i\) changes modulate intracellular Ca\(^{2+}\) buffering. If pH\(_i\) modulates Ca\(^{2+}\)-binding equilibria in cytosolic proteins and/or mitochondrial and ER Ca\(^{2+}\) accumulation, the effects of NH\(_4\)Cl should be observed in the absence of plasma membrane Ca\(^{2+}\) fluxes. To assess the role of intracellular mechanisms, cells were loaded for 30–45 min with both the caged Ca\(^{2+}\) compound NP-EGTA AM and the single-wavelength Ca\(^{2+}\) indicator dye Fluo-4 AM. The superfusing saline was nominally Ca\(^{2+}\)-free and supplemented with 3 mM EGTA (reducing [Ca\(^{2+}\)] to subnanomolar levels). Then 1.5 mM La\(^{3+}\) was included to antagonize voltage-operated channels, store-operated Ca\(^{2+}\) channels, and PMCA (39, 66). Fig. 6A illustrates a recording from a rod photoreceptor showing that flash photolysis of NP-EGTA stimulated abrupt increases in [Ca\(^{2+}\)] in both the outer and inner segments. [Ca\(^{2+}\)] in the outer segment returned rapidly to baseline levels due to subsequent activation of the Na\(^+\)/Ca\(^{2+}\)/K\(^+\) exchanger but PMCA-mediated [Ca\(^{2+}\)] recovery in the inner segment was blocked by the inclusion of La\(^{3+}\). Although plasma membrane Ca\(^{2+}\) fluxes (through voltage-operated, Orai/TRP/store-operated channels, and PMCA) were blocked in these experiments, NH\(_4\)Cl still evoked sustained decreases in [Ca\(^{2+}\)] in the inner segment. In five rods, ΔF/F decreased from 0.955 ± 0.008 in control to 0.3410 ± 0.0638 in the presence of NH\(_4\)Cl (P < 0.002; two-tailed paired t-test; Fig. 6, A and C). A similar effect of NH\(_4\)Cl was observed in cone inner segments (n = 11, P = 0.002; two-tailed paired t-test) (Fig. 6, B and C). It is noteworthy that Na\(^+\) acetate, an acidifying agent, increased [Ca\(^{2+}\)] under these conditions (Fig.
were indistinguishable from those observed in control cells expressing different plasma membrane Ca\(^{2+}\) signals in both the outer segment and inner segment, which is blocked by 1.5 mM La\(^{3+}\). With all IS plasma membrane influx/extrusion mechanism blocked, NH\(_4\)Cl still evoked a reversible decrease in [Ca\(^{2+}\)]. Subsequent permeabilization with ionomycin rapidly decreased [Ca\(^{2+}\)].

Intracellular buffering in photoreceptor inner segments. To obtain an estimate of the amount of intracellular protons bound by diffusing NH\(_3\) and the total intracellular buffering power (the capacity of the cytosol to resist changes in pH) in the photoreceptor inner segment under our experimental conditions, we applied the Henderson-Hasselbach equation:

\[
\frac{[\text{NH}_3]}{[\text{NH}_4^+]^\text{pK}_D} = 10^{\text{pH}_{\text{io}} - \text{pK}_D}
\]

with pK\(_D\) for the weak base (~9.25 for NH\(_4\)Cl). Assuming [NH\(_3\)]\(_i\) is equal to [NH\(_3\)]\(_o\).

NH\(_4\)Cl-induced decrease in [Ca\(^{2+}\)] is blocked by BAPTA but not by EGTA. If Ca\(^{2+}\) and protons compete for binding sites on cytosolic proteins (6–7, 15, 19, 14, 18, 32–33, 36, 51), the effects of the ammonium prepulse should be outcompeted by a fast buffer such as BAPTA. After acquisition of an initial response to NH\(_4\)Cl, the valve regulating solution inflow into the recording chamber was turned off and the saline in the chamber was replaced by an equal volume of saline containing 50 \(\mu\)M BAPTA AM. After 5 min, the flow through the channel resumed, and a transient decrease in the 340-to-380 ratio caused by sequestration of cytosolic Ca\(^{2+}\) during deesterification of BAPTA-AM was typically observed. Following deesterification, [Ca\(^{2+}\)] levels stabilized over the next few minutes. As illustrated in Fig. 7, bath application of NH\(_4\)Cl evoked little change in [Ca\(^{2+}\)], following incubation with BAPTA (n = 3 rods and 3 cones; \(P = 0.1415\), paired t-test). A separate series of experiments in BAPTA-treated cells loaded with BCECF-AM established that pH\(_i\) responses to NH\(_4\)Cl were indistinguishable from those observed in control cells (n = 5; Fig. 7B). These data show that fluorescence of the pH\(_i\) indicator dye is independent of NH\(_4\)Cl-induced changes in [Ca\(^{2+}\)], and suggest that alkalinization modulates intracellular Ca\(^{2+}\) buffering in photoreceptor cytosol.

Intracellular buffering in photoreceptor inner segments. To obtain an estimate of the amount of intracellular protons bound by diffusing NH\(_3\) and the total intracellular buffering power (the capacity of the cytosol to resist changes in pH) in the photoreceptor inner segment under our experimental conditions, we applied the Henderson-Hasselbach equation:

\[
\frac{[\text{NH}_3]}{[\text{NH}_4^+]^\text{pK}_D} = 10^{\text{pH}_{\text{io}} - \text{pK}_D}
\]

with pK\(_D\) for the weak base (~9.25 for NH\(_4\)Cl). Assuming [NH\(_3\)]\(_i\) is equal to [NH\(_3\)]\(_o\).

\[\text{BAPTA AM} \]

\[\begin{align*}
\text{ratio} & = 0.24, 0.12, 0.06, 0.04, 0.02, 0.01, 0.005, 0.002, 0.001, 0.0005, 0.0001
\end{align*}
\]

A test pulse of NH\(_4\)Cl in 20 mM KCl saline caused a decrease in [Ca\(^{2+}\)], followed by a rebound. Next, saline superfusion of the chamber was stopped and the solution in the chamber replaced with a 50 \(\mu\)M BAPTA-AM containing saline. [Ca\(^{2+}\)] exhibited a transient decrease followed by a [Ca\(^{2+}\)] increase slightly above the control value. Exposure of BAPTA-filled cell to NH\(_4\)Cl had no effect on [Ca\(^{2+}\)]. B: pH\(_i\) measurement in a BCECF/BAPTA-loaded rod IS shows the typical pH\(_i\) profile.
for $[NH_4^+]_o = 15$ mM, $pH_o = 7.6$, and $pH_i = 7.2$, $[NH_4^+]_i = \Delta[H^+] = 37.7$ mM (a slight overestimate because binding of $H^+$ changes $pH_i$; 46). Therefore, 15 mM NH$_4$Cl will remove 37.7 mM proton equivalents from the cytosol, change pH by 0.8 units and decrease $[Ca^{2+}]$, by $\sim 100$ nM (Fig. 2D). From the definition of the total intracellular proton buffering capacity ($\beta_T$) (12, 53).

$$\beta_T = \frac{\Delta [H^+]}{\Delta pH_i} = \frac{37.7 \text{ mM}}{0.8 \text{ pH units}} = 47.7 \text{ mM}(\text{pH unit})^{-1} \quad (4)$$

This calculation is similar to the 50 mM/(pH unit)$^{-1}$ obtained by Liebman et al. (45) for toad rod outer segments and higher than $\sim 24$ mM/(pH unit)$^{-1}$ calculated previously for salamander rod outer segments (59). This estimate is also higher than values reported for rat Purkinje cells [10.7 mM/(pH unit)$^{-1}$; 25] and cortical neurons [27 mM/(pH unit)$^{-1}$; 35] but roughly equivalent to 60 mM/(pH unit)$^{-1}$ measured in cardiomyocytes (74–75). High $\beta_T$ is consistent with the need for proton regulation in metabolically active cells such as photoreceptors.

**DISCUSSION**

Our data suggest that relatively small changes in intracellular pH profoundly affect the steady-state [Ca$^{2+}$], in rods and cones. Intracellular alkalinization depolarized the plasma membrane, facilitated Ca$^{2+}$ release/sequestration from Ca$^{2+}$ stores in rod inner segments, and altered the Ca$^{2+}$-H$^+$ equilibrium in the cytosol.

$pH_i$ modulates intracellular signals in rods and cones. It is well known that fixed charges on the surface of the membrane can profoundly affect the measured current-voltage relationship by changing its shape/position on the voltage axis (4). If the change in $pH_i$ evoked by the ammonium pulse primarily modulates membrane surface charge, alkalinization should cause a hyperpolarizing shift in the activation of voltage-dependent ion channels due to sequestration of protons bound to negative fixed charges on the inner leaflet by newly arrived NH$_3$. Lowering the activation threshold in this way would be predicted to enhance Ca$^{2+}$ influx at membrane potentials up to the potential at which L-type Ca$^{2+}$ currents are fully activated in photoreceptors (ca. $-20$ mV). Additionally, the peak amplitude of L-type Ca$^{2+}$ currents is augmented by intracellular alkalinization (e.g., 52, 67, 70–71). Ca$^{2+}$ influx through L-type Ca$^{2+}$ currents should therefore increase during the ammonium pulse, but [Ca$^{2+}$], in depolarized photoreceptors decreased in a dose-dependent fashion with increasing ammonium concentration. Thus, L-type channels are unlikely to represent the primary target of intracellular alkalinization.

Intracellular alkalinization evoked [Ca$^{2+}$], decreases under closed system conditions when the major Ca$^{2+}$ influx and clearance mechanisms in inner segments were blocked. The most parsimonious interpretation of our data, supported by direct measurements of Ca$^{2+}$ activity with ion selective micro-electrodes in other cell types (6–7, 14, 51–52, 74), is that alkalinization regulates reciprocal Ca$^{2+}$-H$^+$ interactions by displacing protons from intracellular Ca$^{2+}$ binding sites (6, 15, 19, 36). The 99% of Ca$^{2+}$ that enters the cytosol immediately binds to phospho, carboxyl, and imidazole groups and the high-affinity helix-loop-helix EF hand motifs of cytosolic proteins (47, 50, 58). Carboxylic Ca$^{2+}$-binding sites are normally protonated so that the protein diffuses within the cytosol as a neutral complex. Ca$^{2+}$ binding lowers $pH_i$ through exchange with H$^+$ at intracellular sites (32, 74–75), whereas protons antagonize the binding of Ca$^{2+}$ at all four calmodulin EF hands (19, 33). Accordingly, injection of Ca$^{2+}$ into invertebrate photoreceptors is equivalent to injection of protons (14, 51, 74). Consistent with $pH_i$ modulation of cytosolic Ca$^{2+}$ buffering, the effect of alkalinization was antagonized by the fast Ca$^{2+}$ buffer BAPTA. The targeting of cytosolic buffering systems is further suggested by the uniformity of spatial [Ca$^{2+}$], changes in alkalini perikaryal, myoid, and ellipsoid inner segment regions, which can be contrasted to highly polarized activation patterns of voltage-operated, store-operated, mitochondrial, and ryanodine/SERCA mechanisms in salamander photoreceptors (39, 41, 65, 69).

We considered the possibility that decreases in [Ca$^{2+}$], were caused by artefactual alkalinization-evoked shifts in the emission of fura-2 and Fluo-4 indicator dyes. However, direct measurements of fura-2 emission showed no artefactual contribution of NH$_4$Cl (Fig. S1). pH changes in the alkaline direction have been demonstrated to have little or no effect on fura-2 $K_d$ (44, 49, 54). Intracellular alkalinization is expected to increase the fura-2 ratio (44), whereas the opposite was observed experimentally. Changes in [Ca$^{2+}$], had no effect on fluorescence properties of the $pH_i$ indicator BCECF.

Intracellular alkalinization evoked [Ca$^{2+}$], increases in a subset of rod photoreceptors. These [Ca$^{2+}$], responses were always transient, mirrored the shape of caffeine-induced [Ca$^{2+}$], increases in depolarized rods (40–41), and were accordingly antagonized by SERCA inhibitors CPA and thapsigargin. We conclude that an increase in $pH_i$ modulates Ca$^{2+}$ release from internal stores in rod perikarya, possibly by regulating the open probability of ryanodine channels (2, 18, 20, 79). In contrast to the well-understood pH sensitivity of voltage-operated channels (3, 52, 67, 59, 70), PMCA s (61, 72), and Ca$^{2+}$ stores (2, 79), little is known about $pH_i$ sensitivity of neuronal SOCE. Our results regarding photoreceptor SOCE are inconclusive. While the amplitude of SOCE in the majority of cells appeared to be unaffected by alkalinization, the variability was high, and significant subsets of alkalini sed cells showed increased or decreased levels of [Ca$^{2+}$],. Such results may be expected if photoreceptors express several different classes of TRPC/Orai channels (e.g., 65). Parenthetically, store-operated responses from Muller glial cells recorded alongside rods/cones were consistently suppressed by intracellular alkalinization (n = 9; P. Barabas and D. Križaj, unpublished observations). This observation argues for SOCE mechanisms that are mediated by different Ca$^{2+}$-permeable channels in different retinal cells.

**HEPES and pH buffering in photoreceptors.** Studies of the blood acid-base balance in ectothermic amphibians such as *Ambystoma* suggest that the concentration of bicarbonate in arterial blood can vary widely over the physiological temperature range of 5 to 25°C (13a, 14a). Hence, HEPES has
typically been the buffer of choice in studies that characterized pH, chloride, and calcium signaling mechanisms in isolated photoreceptors (3–4, 17, 34, 37, 39–41, 50, 55, 60, 63–66, 68–69) and horizontal cells (67) and has also been used in investigations of synaptic transmission at photoreceptor synapses (28, 37). In the present study, the main mechanism regulating cytosolic acid loads under nominally bicarbonate-free conditions was the ubiquitous electroneutral Na+/H+ antiporter, which couples proton transport to the sodium gradient (34, 59). Vertebrate photoreceptors may also express electroneutral Cl-/HCO₃⁻ exchange (28, 34, 38, 59) and electrogenic Na⁺/HCO₃⁻ cotransport and Na⁺-driven C-/HCO₃⁻ exchange mechanisms (10, 83), which could be functionally coupled to carbonic anhydrases within the bicarbonate transport metabolon (62). It is likely that addition of bicarbonate and activation of cytosolic and extracellular carbonic anhydrases has additional effects on photoreceptor pH, and [Ca²⁺]i regulation, outer retinal feedback, and photoreceptor cell death (10, 23, 83). For example, switching from bicarbonate-based to HEPES-buffered saline dramatically increased the rate of photoreceptor neurotransmitter release and the amplitude of light-induced responses of horizontal cells in the intact salamander retina (28), suggesting that loss of bicarbonate alkalinizes salamander photoreceptors. In contrast, application of 20 mM HEPES acidified and hyperpolarized horizontal cells and reduced the amplitude of light-induced responses in teleost and rabbit retinas (23, 27). While pH, was shown to modulate gap-junctional permeability in horizontal cells (23), the properties of voltage-operated, store-operated, and cytosolic Ca²⁺ buffering mechanisms in teleost/rabbit photoreceptors remain to be characterized.

Functional significance. pH, measurements revealed that photoreceptors in HEPES-buffered saline are considerably more acidic than if protons were in electrochemical equilibrium. The average pH, in rod and cone inner segments ranged from 7.15 to 7.25, a marked reduction from the external saline pH of 7.6. This result is consistent with observations from most animal tissues in which ATP hydrolysis and glycogenolysis (i.e., lactate production) provide continuous acid load (e.g., 11, 28, 38, 80, 82). In cells depolarized by 20 mM KCl, a decrease in pH, by ~0.4 units caused ~90 nM increase in inner segment [Ca²⁺]i. The physiological impact of metabolically generated protons would be to stabilize [Ca²⁺]i levels, increase the presynaptic [Ca²⁺]i range, and minimize the probability of regenerative activation of voltage-operated ICa in the inner segment (1). This effect may be emphasized in the presence of endogenous bicarbonate, which tends to further acidify cells through Cl-/HCO₃⁻ mechanisms (25, 28, 81).

It remains to be determined to what extent managing sustained metabolically induced acidosis and/or changes in [Ca²⁺]i is important for photoreceptor survival. Excessive changes in pH, and/or [Ca²⁺]i have been shown to compromise vital neuronal functions. Intracellular acidification during ischemia and hypoxia can suppress Na⁺/K⁺ and Na⁺/Ca²⁺ exchange, increase extracellular K⁺ concentration, and trigger [Ca²⁺]i elevations in many types of neurons including photoreceptors (30–31, 42). Combining acidosis with anoxia both increases and prolongs [Ca²⁺]i elevations (30). Importantly, photoreceptors from mice with dysfunctional sodium-bicarbonate transporters may degenerate, in part, due to secondary pathological changes in [Ca²⁺]i (10, 83). In conclusion, this study suggests that pH, changes in photoreceptor cells cannot be disentangled from concomitant changes in [Ca²⁺]i because protons are engaged in an incessant cross-talk with multiple intracellular Ca²⁺ signaling pathways. Reciprocal pH, [Ca²⁺]i interactions represent a signal through which the metabolic activity of the cell adjusts its activity-dependent messenger signals.

ACKNOWLEDGMENTS

We thank Drs. David Copenhagen (University of California San Francisco) and Mitchell Chesser (New York University) for comments on early versions of the manuscript.

GRANTS

This work was supported by The National Eye Institute Grants EY-13870, P30-EY-014800, EY-10542, The International Retina Research Foundation, Foundation Fighting Blindness, and the Moran TIGER award. The research was also supported by unrestricted grants from Research to Prevent Blindness to the Moran Eye Institute at the University of Utah, and the Department of Ophthalmology and Visual Sciences at the University of Nebraska Medical Center.

DISCLOSURES

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

REFERENCES


AJP-Cell Physiol • VOL 300 • JANUARY 2011 • www.ajpcell.org


