Regulation of intracellular pH in salamander retinal rods

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1. We measured intracellular pH (pHi) in rods isolated from the retina of the axolotl salamander, Ambystoma mexicanum, using the fluorescent indicator 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF).

2. The light exposures associated with data acquisition had no marked effect on pHi. There was no sharp change between the value obtained from the first exposure of dark-adapted rods and subsequent readings. Increasing the acquisition frequency from 1 to 10 min⁻¹ either had no effect, or brought about a slow acidification, which was stopped or reversed when the low frequency was restored.

3. In nominally HCO₃⁻-free solution at pH 7.5, the rods had a steady-state pHi of 7.09 ± 0.02 (n = 46) and a buffering power (β₁) of 24 ± 1 mM (pH unit)⁻¹ (n = 48). The buffering power was virtually constant in the pH range 6.6–8.0. In the same range, pHi depended linearly on perfusion pH (pHᵢ) with regression coefficients of 0.4–0.5.

4. There were no significant differences between the inner and outer segment of intact rods as regards steady-state pHi, or responses to experimental treatments.

5. Recovery from an intracellular acid load imposed by sodium propionate or an NH₄Cl prepulse in nominally bicarbonate-free perfusate was completely blocked by decreasing the extracellular Na⁺ concentration to 7 mM, and slowed by 86% by applying 1 mM amiloride.

6. Introduction of 2% CO₂–13 mM HCO₃⁻ caused an alkalinization that was often preceded by a transient acidification. Steady-state pHi was on average 0.1 pH units higher than in nominally bicarbonate-free solution. The mean acid extrusion rate, calculated on the assumption that CO₂–HCO₃⁻ behaves as an open system, was 19% higher (31 ± 2 mM h⁻¹) than in a solution buffered only by HEPES (26 ± 2 mM h⁻¹).

7. In the presence of CO₂–HCO₃⁻, 100 μM 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) decreased the acid extrusion rate by 20% on average. Lowering the extracellular Cl⁻ concentration to 7 mM raised pHi, but did not significantly affect the acid extrusion rate.

8. We conclude that retinal rods regulate pHi by both Na⁺–H⁺ exchange and mechanism(s) involving HCO₃⁻–Cl⁻ exchange. In the present conditions, the Na⁺–H⁺ exchanger appears as the dominant mechanism for acid extrusion.

Phototransduction in vertebrate photoreceptor cells is sensitive to pH. Early work showed that the amplitude of mass rod responses from the frog retina dropped by 70% when perfusion pH (pH₀) was decreased from 8 to 6 (Sillman, Owen & Fernandez, 1972), and that this was associated with a decrease of the Na⁺ conductance of rod outer segments (Gedney & Ostrow, 1978; Wormington & Cone, 1978). In current recordings from isolated frog rods exposed to pulses of low-pH perfusion, Liebman, Mueller & Pugh (1984) found a slowing down of response kinetics and a rapid depression of the dark current with a monotonic pH dependence in the range from 6 to 3.5. Injecting H⁺ into the rods by using weak acids or NH₄Cl prepulses, they demonstrated that at least part of the action is internal, due to changes in intracellular pH (pHi). Some targets of H⁺ action in rods have been experimentally identified. The Na⁺ conductance of inside-out patches of outer segment membrane is halved when perfusion pH is 5.5 compared with perfusion at neutral pH (Menini, 1989), and phosphodiesterase activity falls gradually when pH is decreased from pH 8 (Miki, Baraban, Keirns, Boyce & Bitensky, 1975). Particularly interesting is the decrease in the rate of Na⁺–Ca²⁺ exchange with acidic perfusion (a 5-fold drop on going from pH₀ 7.5 to pH₀ 6.1; Hodgkin & Nunn, 1987), which couples H⁺ to the main effector for light adaptation, Ca²⁺. At least part of this effect seems to be internal, mediated by a change in pH (Yoshikami & Hagins, 1984).
The pH sensitivity of phototransduction should be seen in the context of the heavy and fluctuating loads of metabolic acid (CO$_2$ and lactic acid) to which photoreceptors are exposed. The retina has the highest energy production of all vertebrate tissues, and in photoreceptor cells, a large part of the energy is provided by glycolysis (Winkler, 1981; Hsu & Molday, 1994). In addition, phototransduction itself involves substantial H$^+$ release from cGMP turnover (Dawis, Graeff, Heyman, Walseth & Goldberg, 1988; Pugh & Lamb, 1990). Rods would a priori be expected to need a high buffering power and efficient mechanism(s) for acid-base transport to regulate pH$_1$ and light sensitivity. In retinas kept in media of low buffering capacity, significant decreases in rod current and [cGMP] are observed (Winkler, 1986; Meyertholen, Wilson & Ostroy, 1986). The special role of CO$_2$–HCO$_3^-$ buffer, which has been found to increase [cGMP] and the light-sensitive current and accelerate response kinetics (Lamb, McNaughton & Yau, 1981; Lamb, 1984; Meyertholen et al. 1986; Donner, Hemilä, Kalamkarov, Koskelainen & Shevchenko, 1990b) is consistent with the idea that bicarbonate helps to prevent intracellular acidification.

Thus, photoreceptors are neurons for which much is known about the effect of pH on the main physiological functions, as well as about acid loads and activity-related fluctuations in pH$_1$ in the normal in vivo environment (Oakley & Wen, 1989; Borgula, Karwoski & Steinberg, 1989; Yamamoto, Borgula & Steinberg, 1992). Until now, however, there is only electrophysiological evidence to suggest how the photoreceptors achieve the necessary control of pH$_1$ to transduce light signals reliably. Experiments applying inhibitors and ionic substitutions chosen to affect putative acid–base transport mechanisms have revealed changes in the dark current and response kinetics consistent with predicted changes in pH$_1$ (Donner et al. 1990b; Katz & Oakley, 1990; Koskelainen, Donner, Kalamkarov & Hemilä, 1994).

In the present work, we study transport mechanisms and buffering power in isolated salamander rods, measuring pH$_1$ by a microfluorometric technique. The preparation is basically identical to that used in electrophysiological suction-pipette studies, allowing correlation of the present pH$_1$ measurements with measurements of the light-sensitive current of functioning rods (Lamb, 1984; Liebman et al. 1984; Koskelainen et al. 1994).

We find that the pH$_1$-buffering power of rods is not exceptionally high, and is in the range reported for other neurones. The cells extrude acid by at least two types of mechanism. In the nominal absence of HCO$_3^-$ ions, rods recover from imposed acid loads by a Na$^+$-dependent, amiloride-sensitive mechanism, consistent with a Na$^+$–H$^+$ exchanger. In the presence of CO$_2$–HCO$_3^-$, acid extrusion rates are somewhat higher and sensitive to the anion-transport inhibitor DIDS. Lowering extracellular Cl$^-$ causes a transient alkalization, implicating HCO$_3^-$–Cl$^-$ exchange. In our experimental conditions, however, the Na$^+$–H$^+$ exchanger appears as the dominant mechanism for acid extrusion.

### METHODS

#### Dissociation and loading of cells

Larval axolotl salamanders (Ambystoma mexicanum) were obtained from a breeder in Moscow, Russia. Dark-adapted animals were decapitated and fully pithed both above and below the decapitation, and both eyes were enucleated. The retinas were isolated in standard Ringer solution (see below). One half-retina was transferred to a small (100 µl) volume of the same solution in the perfusion chamber and shredded to small pieces. The other half and the second retina were stored refrigerated in darkness and could be successfully used several hours after dissection. The bottom of the perfusion chamber was formed by a coverslip precoated with Cell-Tak® adhesive (BioPolymers, Inc., USA) serving to anchor the dissociated cells. Larger pieces of retina were removed. All preparatory procedures were carried out in dim red light. The cells were then incubated for 15–20 min in darkness in a 10 µM solution of the membrane-permeant acetoxyethyl ester of the indicator dye 2',7'-bis(carboxyethyl)-5-(and -6)carboxyfluorescein (BCECF AM; Molecular Probes, Inc.). After loading, cells were superfused with standard Ringer solution for a further 20 min in darkness before measurements were started. The flow rate through the trough-shaped perfusion chamber (volume, about 100 µl) was 0.35 ml min$^{-1}$.

When data were averaged from several cells, these were always from the same experiment (the same preparation), lying within a distance of less than 0.5 mm in the specimen chamber, so solution changes were practically simultaneous for all. Some measurements were made on small pieces of retina, which had attached receptor side down to the bottom of the chamber. All experiments were carried out at room temperature (22–25°C).

#### Solutions

The nominally bicarbonate-free standard Ringer solution (Hepes Ringer solution) contained (mM): NaCl, 95; KCl, 3; CaCl$_2$, 0.9; MgCl$_2$, 0.5; glucose, 10; and Hepes, 10. pH was adjusted to 7.5 with NaOH. Before experiments all solutions were supplemented with 5% of Leibovitz culture medium L-15 (Sigma), which in electrophysiological experiments has been found to improve the viability of isolated rods (Koskelainen et al. 1994). Low-Na+ solution was prepared by replacing all NaCl of the standard Ringer solution with N-methyl-1-glucamine chloride (NMG-Cl). Owing to the added L-15, the Na+ concentration of this solution was 7 mM. The bicarbonate-containing solution (bicarbonate Ringer solution) was prepared by replacing 13 mM NaCl with equimolar NaHCO$_3$ and equilibrating the solution with 2% CO$_2$–98% air. In propionate-containing solutions, sodium or NMDG propionate was substituted for NaCl or NMDG-Cl. Solutions containing NH$_4$Cl were prepared by substituting NH$_4$Cl for NaCl or NMDG-Cl. 4,4′-dithiobis-2,2′-disulphonic acid (DIDS; Sigma) was stored as 10 mM stock solutions made in DMSO. Amiloride (Sigma) was dissolved directly in the experimental solution.

#### Data acquisition

The fluorescence imaging system (Photon Technology International Inc., NJ, USA) consisted of an inverted microscope (Nikon Diaphot with a ×20 fluorescence objective), a monochromator-chopper unit, and a silicon-intensified camera (Hamamatsu, Hamamatsu City, Japan). During each data acquisition cycle a pair of fluorescence images of the cells were stored on the hard disk of a microcomputer, one (pH-sensitive) obtained with 495 nm excitation (fluorescence intensity denoted $I_{495}$), another (pH-insensitive) with 440 nm (fluorescence intensity denoted $I_{440}$). The emission light (peaking at 525 nm) passed through an edge filter transmitting wavelengths above 505 nm before reaching the camera. The intensity of the 495 nm excitation light was ca 50 µW cm$^{-2}$.
estimated to produce somewhat less than $10^7$ photosomizations per second in a salamander rod with the dark-adapted density of native visual pigment. (We assumed that: the pigment is a mixture of rhodopsin$^\text{S^29}$ and porphyropsin$^\text{b}$ with a peak light sensitivity of 512–516 nm; specific absorbance at $\lambda_{\text{max}}$ is 0.01 $\mu$m$^{-1}$ for transverse incidence of unpolarized light; rod outer segments are 10 $\mu$m thick (Hárosi, 1975; unpublished microspectrophotometrical observations by V. I. Govardovskii, N. Fyhrquist & K. Donner); and the quantum efficiency for isomerization is 0.67 (Dartnall, 1972)).

The 440 nm light was estimated to produce isomerizations at about 30% of this rate. In a typical experiment (where 4 video frames of 30 ms each were averaged at both excitation wavelengths), the light exposure associated with one acquisition cycle lasted for 1.7 s (0.85 s of 495 nm exposure directly followed by 0.85 s of 440 nm exposure); otherwise the light path was closed by a computer-controlled shutter. In a dark-adapted rod with full pigment complement, one such (double) exposure would produce about $10^7$ isomerizations. Assuming that a dark-adapted rod contains about $3 \times 10^9$ molecules of visual pigment, one cycle would isomerize 0.3–0.5% of the pigment. Our usual data acquisition interval was 15 s. If no pigment regeneration occurs, 50–70% of the pigment would be in the bleached state at the end of a typical 1 h experiment.

In the experiments where we wanted to measure steady-state pH$_i$, in a situation as close as possible to the dark-adapted state, sampling was restricted to one single video frame (at both wavelengths) per cycle in order to limit the time of illumination that could have affected the first reading. Allowing for the shutter delay (time for opening) of 100 ms before data sampling starts, acquisition of the pH-sensitive fluorescence image was completed within 130 ms of the onset of illumination. This sets upper limits of $\approx 10^6$ isomerizations per rod (< 0.05% of the pigment) for the amount of light and 130 ms for the time of illumination that may have affected the first pH$_i$ value obtained from a previously dark-adapted rod (see the first section of the Results).

Analysis of the data

A macro program read the images pairwise from the hard disk, formed pixel-to-pixel fluorescence ratio values, and calculated the median ratio values inside rectangular 'regions of interest' that were defined on selected cells in the stage of data analysis. The background fluorescence was low (less than 5% of the fluorescence of cells) and independent of experimental conditions, and therefore had negligible effect on fluorescence ratios. The $I_{495}/I_{440}$ ratio values were converted to pH$_i$ values using a calibration curve obtained by bathing the cells in nigericin-containing, high-K$^+$ solution (Thomas, Buschbaum, Zinniak & Racker, 1979). In the presence of nigericin, pH$_i$ rapidly equilibrates with pH$_o$, making it possible to vary pH$_i$ simply by varying pH$_o$. The fluorescence ratio values were normalized, taking the ratio at pH 7.0 as unity, plotted as a function of pH$_i$, and fitted to the equation:

$$\frac{I_{495}}{I_{440}} = \frac{10^{pH_i-pK} + A}{B(1 + 10^{pH_i-pH_P})},$$

where $A$ and $B$ are constants and $pK$ is the apparent ionization constant of the indicator in the cell.

In some experiments the nigericin calibration was checked by using the null-point method described by Eisner, Kenning, O'Neill, Pocock, Richards & Valdeolmillos (1989). Cells were briefly super-surfused with solutions containing tetrathylamine (TMA) and butyrate in different proportions. If pH$_i$ is exactly the same as the nominal pH of the calibration solution, the effects of butyrate and TMA cancel out and pH$_i$ remains unchanged. If pH$_i$ is lower than the nominal pH, the cell gets more alkaline; if pH$_i$ is higher, it becomes more acidic. By using three or four solutions, the initial pH$_i$ levels could be determined with an accuracy of 0.05 pH units.

Results are given as means ± S.E.M. The statistical significance of differences of means was tested with Student's t test.

RESULTS

Intracellular pH in the absence of HCO$_3^-$

Effect of light stimulation

With fluorometric techniques, the physiological state of a photosensitive cell like a retinal rod will inevitably be affected by the measurement itself. We studied the effect of the light exposures on pH$_i$ by varying the data acquisition frequency between 1 and 10 min$^{-1}$.

At the beginning of experiments rods were dark adapted, presumably with part of the light-sensitive channels open and a membrane potential of about −40 mV (e.g. Baylor & Nunn, 1986). However, after the very first light exposure rods must have been essentially saturated throughout the experiment, i.e. hyperpolarized below −40 mV with the light-sensitive channels closed. This would be true even with our lowest data acquisition frequency of 1 min$^{-1}$, which (in terms of a steady rate) delivers 1.7 $\times$ 10$^5$ isomerizations per rod per second (see Methods). In the intact frog retina, the light-sensitive current drops below 10% under a steady illumination of 10$^5$ isomerizations per second (Donner, Koskelainen, Djupsund & Hemiš, 1995). Isolated rods are likely to be even more strongly saturated, as they light-adapt less well (see Donner, Copenhagen & Reuter, 1990a). Thus, increasing the acquisition frequency to 10 min$^{-1}$ increased the rate of phototransduction reactions inside the cell, but probably did not significantly change the membrane potential, permeability, or circulating current.

Figure 1, in which data from several rods and rod outer segments recorded in two experiments is assembled, illustrates the range of behaviour observed. Remember that the first exposure was given to cells that had never been exposed to strong light and had been in darkness for at least 20 min. Yet there was no clear difference between the pH$_i$ value obtained with the first light stimulus and those obtained with subsequent stimuli.

Although a light exposure delivering 10$^7$ isomerizations s$^{-1}$ (as estimated for the 495 nm stimulus; see Methods) shuts off all the light-sensitive current (Lamb & Pugh, 1992) in less than the 100 ms from the moment the shutter starts opening to the beginning of data acquisition, the first pH$_i$ reading is not likely to have been significantly affected by illumination. Closure of the light-sensitive conductance, or hyperpolarization of the plasma membrane, can cause no significant redistribution of H$^+$ across the membrane in the 130 ms needed to complete the measurement of the pH-sensitive signal. What remains is H$^+$ liberation from cGMP hydrolysis. Over this short time scale, hydrolysis will essentially be limited to the existing 'dark' pool of free cGMP.
for which Pugh & Lamb (1990) give an upper-bound estimate of 9 μM. With a buffering power of 24 mM (pH unit)\(^{-1}\) (see below), complete hydrolysis would displace pH\(_i\) by less than 0·001 pH units. Thus we think that the initial pH\(_i\) values in Fig. 1 are representative of dark-adapted rods.

About 15 min into the experiments, the initial data acquisition frequency of 1 min\(^{-1}\) was increased to 10 min\(^{-1}\) for a period of about 15 min. This caused no abrupt shifts. The most common response was a slow, steady acidification, which could sometimes be quite significant (trace \(d\)). However, decreasing the stimulation frequency back to 1 min\(^{-1}\) again invariably brought a change in a more alkalinizing direction (\(\text{relative to the preceding trend}\)), even when no acidification had occurred during the high-frequency stimulation (traces \(b\) and \(c\)). Acidification always stopped, and in most cases a slow, steady alkalinization started. pH\(_i\) in the outer segment of intact rods followed the changes in the inner segment quite closely (traces \(b\) and \(c\)). In detached outer segments, absolute pH\(_i\) was markedly higher than in intact rods, but there was no obvious difference in the response to light stimulation (trace \(a\)).

**The outer vs. inner segment of intact rods: emission intensity and pH\(_i\)**

Both excitation (495 and 440 nm) and emission (peak at 525 nm) wavelengths are in the range where the visual pigment mixture, which has its peak light sensitivity at 512–516 nm, has a high absorption. This will in principle decrease emission intensities in the outer segment and might even cause spuriously low \(I_{495}/I_{440}\) ratio values, as the 495 nm excitation light will be absorbed more strongly than the 440 nm light.

Indeed, the absolute fluorescence intensity in the outer segment was 2–3 times lower than in the inner segment of the same rod, both during calibrations with nigericin and in actual experiments. The \(I_{495}/I_{440}\) ratios, however, were not significantly different, and remained equal during long experiments in spite of substantial bleaching of visual pigment (see Methods).

Even the lower absolute fluorescence intensities in the outer segment are probably not due mainly to absorption by the visual pigment. We estimate that near the beginning of experiments less than 20% of transversely incident 495 nm light and roughly the same proportion of emitted light would be absorbed, and these proportions would be lower towards the end, with much of the visual pigment in the bleached state. The low outer-segment fluorescence thus seems to be determined largely by other factors. One possibility is differential distribution of the dye; e.g., the dense stack of lipid discs in the outer segment might significantly decrease the proportion of (cytoplasmic) space accessible to the dye compared with that in the inner segment.

**Steady-state pH\(_i\)**

In nominally bicarbonate-free solution at pH 7·5, the mean initial pH\(_i\) was 7·09 ± 0·02 (\(n = 46\)). There was no significant

![Figure 1. Effect of the data acquisition (light stimulation) interval on pH\(_i\)](image)

During the first 10 min the data acquisition interval was 60 s. It was then shortened to 6 s for 15 min, and finally returned again to 60 s. Trace \(a\) shows the mean of recordings from five detached outer segments, traces \(b\) and \(c\) represent the inner and outer segment, respectively, of one intact rod, and trace \(d\) the mean of the inner segments of three other rods.
difference between the inner and outer segment of intact rods (t test for paired values). At a membrane potential of −40 mV, H⁺ would be in electrochemical equilibrium at pH_i = 7·5 − 40/58 = 6·81, which is significantly lower than the measured mean value. If the membrane potential is more negative (as it would normally be in saturated rods), the difference would be even greater. This indicates that the rods are continuously extruding H⁺.

Dependence of pH_i on pH_o

When pH_o was stepped up or down, pH_i changed in the same direction, exponentially approaching a new steady level. The change of pH_i varied from cell to cell, but was always less than half of the change of pH_o. The dependence of steady-state pH_i on pH_o was approximately linear in the pH_o range 6·5–8·0 with regression coefficients in the range 0·4–0·5. Thus, pH_i did not passively follow changes in pH_o. If it is assumed that the membrane potential remained constant, the difference between measured and equilibrium pH_i values increased when pH_o was lowered. At pH_o 7·0, pH_i would already have been at least 0·6 units higher than the calculated equilibrium value.

Buffering power

The intracellular buffering power was determined by exposing the rods to stepwise increasing or decreasing concentrations of a weak acid (5, 10, 20 and 50 mM propionate in low-Na⁺ solution) or a weak base (1, 2·5, 5 and 10 mM TMA-Cl, or 10 and 20 mM NH₄Cl). The intrinsic (non-bicarbonate) buffering power of the cell (β_i) can be calculated from the equation:

\[ \beta_i = \frac{-\Delta[A^-]_i}{\Delta p\text{H}_i} = \frac{\Delta[B^+]_i}{\Delta p\text{H}_i}, \]

where \([A^-]_i\) and \([B^+]_i\) are the concentrations of the weak acid anion and the weak base cation in the cell. Assuming that the non-ionized form of the acid or base equilibrates rapidly across the cell membrane, while the membrane is practically impermeable to the ion form, these concentrations can be calculated using the Henderson–Hasselbalch equation:

\[ [A^-]_i = \frac{c_a \times 10^{p\text{H}_i-pK_a}}{10^{pK_a-pH_o} + 1} \]

and

\[ [B^+]_i = \frac{c_b \times 10^{pK_a-pH_o}}{10^{pK_a-pH_o} + 1}, \]

where \(c_a\) and \(c_b\) are the total concentrations of the weak acid or base in the extracellular solutions, and \(pK_a\) is the acid dissociation constant of the weak acid or base (4·87 for propionic acid, 9·81 for TMA, and 9·25 for NH₄OH).

All three compounds yielded similar values for \(\beta_i\). Linear regression analysis on the pooled data (48 measurements from 6 experiments; Fig. 2) resulted in the equation \(\beta_i = -5·22\ \text{pH}_i + 61·62\), where \(\text{pH}_i\) is the midpoint of the pH range for which \(\beta_i\) was determined. The slope does not

![Figure 2. Relation between the intrinsic (non-bicarbonate) buffering power (\(\beta_i\)) and intracellular pH \(\beta_i\) was calculated from the change in \(\text{pH}_i\) produced by changing the concentration of a weak acid (propionic acid) or a weak base (TMA or NH₄Cl) and plotted as a function of the mean of the initial and final \(\text{pH}_i\) values. The lines represent the linear regression of \(\beta_i\) on \(\text{pH}_i\) and the 95% confidence interval of the regression line.](image-url)
differ significantly from zero \((F = 3.94, P > 0.05)\), and the intracellular buffering power thus showed no significant dependence on \(pH_i\) in the range 6.6–8.0. The mean value was \(\beta_i = 24 \pm 1\) mm (pH unit)\(^{-1}\).

**Recovery from acid loads**

To study the properties of \(pH_i\), regulating mechanisms, rods were experimentally acidified either by addition of sodium propionate or by \(\text{NH}_4\text{Cl}\) prepulses. In the former case, \(pH_i\) drops due to the diffusion of the uncharged acid into cells, where it dissociates into hydrogen ions and negatively charged propionate ions. When \(\text{NH}_4\text{Cl}\) is introduced, on the other hand, free \(\text{NH}_3\) first equilibrates rapidly across the cell membrane and is hydrated inside the cell, liberating \(\text{OH}^-\) ions. This fast alkalinization is followed by a slower acidification phase, usually attributed to the influx of \(\text{NH}_4^+\) ions. In saturated rods, where the outer segment conductance is near zero (Baylor & Nunn, 1986), this influx would mainly be through the inner segment. Endogenous acid production would also contribute to the slow relaxation. When \(\text{NH}_4\text{Cl}\) is withdrawn, \(pH_i\) falls below the initial level because of the extra acid equivalents incurred during the slow phase.

Both with propionate and \(\text{NH}_4\text{Cl}\) the induced acidification was followed by gradual recovery, with \(pH_i\) eventually returning to the original level. In the experiments with propionate, washout of the acid was associated with a rapid \(pH_i\) rise to well above the original steady-state level. After this alkaline overshoot, \(pH_i\) gradually returned to the original level, but (in nominally bicarbonate-free medium) the relaxation was often very slow, sometimes taking more than an hour to complete. These main features can be seen in Fig. 3A, in the response to the second (control) application of propionate and its subsequent washout, i.e. the last 20 min of the record. (Due to the long recovery time, these control measurements were usually made last in a sequence of experimental treatments.)

**Figure 3.** Recovery of \(pH_i\) from an acid load in reduced extracellular \(\text{Na}^+\) concentration and at lowered \(pH_o\).

A, in the beginning of the experiment, three calibration solutions with nominal \(pH\) values of 7.2, 7.0 and 6.9 were applied for about 30 s each. The nominally \(\text{HCO}_3^-\)-free control solution was replaced with a solution containing only 7 mm of \(\text{Na}^+\) (95 mm \(\text{Na}^+\) replaced by NMDG\(^+\)). The rods were then acidified with 50 mm propionate (prop; propionate replacing Cl\(^-\)) in the low-\(\text{Na}^+\) solution, and thereafter in the control solution. The heavy line represents the mean of the inner segments of five rods, and the lighter one the mean of the outer segments of the same rods (the five rods were lying close to each other in the same preparation). The sampling interval was 15 s. B, three \(\text{NH}_4\text{Cl}\) pulses (20 mm) were applied in nominally \(\text{HCO}_3^-\)-free solution. The recovery after the first pulse was in control solution. After the second pulse the rods were first kept in low-\(\text{Na}^+\) solution (no recovery) and then in the control solution with 102 mm \(\text{Na}^+\) (steep recovery). After the third pulse (in control \(\text{Na}^+\)), the initial phase of recovery occurs at \(pH_i = 7.0\) (weak recovery), and the last phase at \(pH_i = 7.5\) (steep recovery). The sampling interval was 8 s. The curve represents the mean of four rods within a small area in the same preparation.
Dependence of the recovery on [Na+]o and pHo
When the Na⁺ concentration of the perfusate ([Na+]o) was lowered from 102 mM to 7 mM (95 mM NaCl replaced with NMDG-Cl), pHr invariably began to fall. In many cells this decrease was quite slow (e.g. Fig. 3A), while in some cells rather an abrupt drop occurred, with pHr falling to 6.5 in a few minutes. When cells were then further acidified by replacing NMDG-Cl with NMDG propionate, there was no recovery as long as propionate was present. After washout of propionate, pHr rose rapidly, but without any alkalinizing overshoot. Restoring normal [Na+]o raised pHr to close to the original steady-state level. When the same rods were subsequently exposed to propionate in control solution, pHr recovered fully, even in the presence of propionate, and washout was accompanied by the usual alkalinizing overshoot.

The effect of low [Na+]o was also evident when the acid load was produced by an NH₄Cl prepulse, as shown in Fig. 3B. If the pulse was followed by superfusion with low-Na⁺ solution, pHr remained low. When [Na+]o was restored to normal, pHr recovered even faster than in the control experiment. A likely explanation is that [Na+]o had dropped while [Na+]o was low, so after return to control solution there was an exceptionally strong inward Na⁺ gradient. The regulation system responsible for pHr recovery after acidification in nominally bicarbonate-free medium thus appears to be absolutely dependent on Na⁺.

In some experiments recovery from an NH₄Cl prepulse was studied at normal [Na+]o, but with pHo reduced to 7.0. As shown in Fig. 3B, recovery was clearly slower when pHo was lower. Raising pHo to 7.5 again increased the rate of recovery back to the control level.

Effect of amiloride
The only known Na⁺-dependent process that can mediate recovery from acid loads in bicarbonate-free solution is Na⁺–H⁺ exchange. To characterize the mechanism further, we performed acid-loading experiments in the presence of amiloride, a well-known inhibitor of Na⁺–H⁺ exchange. Recovery after acidification with an NH₄Cl prepulse was slow as long as amiloride (1 mM) was present, but upon washout of the amiloride the rate immediately became about as fast as in the subsequent control (Fig. 4). The rate of extrusion of acid equivalents was calculated by multiplying the initial recovery rate by the mean buffering power of the rods. In the presence of amiloride the average rate was only 14% of that in the control solution. Thus 1 mM amiloride inhibits the transport mechanism reversibly, but not completely.

In some rods, amiloride superfusion in itself triggered a clear decrease of pHr, suggesting that these cells were under a continuous acid load and that a Na⁺–H⁺ exchanger was actively extruding hydrogen ions even in the steady state. In other experiments, including that shown in Fig. 4, amiloride caused no clear change in steady-state pHr (cf. the highly variable rates of acidification when [Na+]o was decreased, above). The variation is not surprising given that different cells are in different physiological states, varying with respect to metabolic activity (as clearly experienced in electrophysiological recordings from isolated cells). Low metabolism will decrease not only metabolic acid loads, but also those due to cGMP hydrolysis, as cGMP synthesis will be retarded. Generally speaking, steady-state pHr is determined as a dynamic balance of acid production and extrusion, and the same pHr may be associated with quite...
different absolute rates of the opposing processes. When extrusion is inhibited, however, the rate of acidification will reflect the rate of acid production.

**Regulation of pH in the presence of CO₂–HCO₃⁻**

**Effect of introducing CO₂–HCO₃⁻**

When the nominally bicarbonate-free, Hepes-buffered standard superfusate was changed to one containing 13 mM HCO₃⁻ equilibrated with 2% CO₂, pH₁ first dropped and then slowly rose to a level ca 0.1 pH units more alkaline than the initial baseline (Fig. 5). In some cells the alkaline shift occurred without any preceding acid transient. The acidification, which presumably resulted from hydration of CO₂ (CO₂ + H₂O ⇌ H₂CO₃ ⇌ H⁺ + HCO₃⁻) inside the cells, was clearly slower than that produced by propionate, taking more than 2 min to reach the reversal point. This is consistent with the view that there is little or no carbonic anhydrase in retinal rods (Musser & Rosen, 1973). In cells that contain carbonic anhydrase, hydration of CO₂ takes only seconds and the rate of CO₂-produced acidification is determined by the rate of the solution change (Saarikoski & Kaila, 1992).

**Acid extrusion rate in the presence of CO₂–HCO₃⁻**

In Fig. 5, identical propionate pulses were administered first in the absence and then in the presence of added CO₂–HCO₃⁻. There are three notable differences between the responses. First, the initial acidification peak is smaller in bicarbonate-containing Ringer solution. Second, the later phases of recovery from acidification are steeper, in spite of the fact that acid extrusion in the bicarbonate-free situation is supported by a stronger outward gradient for H⁺ (larger difference between pH₂ and pH₁). Third, the descent to baseline after the washout-induced alkalinization is faster.

In Fig. 5, this is obvious only for the isolated rod, but it is borne out also by the fast relaxation of the alkalinizations after propionate withdrawal in Fig. 7 (below).

The acid extrusion rate is the product of the rate of pH₁ change and the total buffering power of the cell. When comparing rates of acid extrusion with and without CO₂–HCO₃⁻, the bicarbonate buffering power must be taken into account. The additional buffering power due to CO₂–HCO₃⁻ (βₜ) is directly proportional to the HCO₃⁻ concentration in the cell:

\[ β_{CO_2} = 2.3 \times [HCO_3^-] = 2.3 \times 10^{pH_1-pK'} \times α \times P_{CO_2}, \]

where \( P_{CO_2} \) is the partial pressure of CO₂ (atm), \( α \) is the solubility of CO₂ (mmol l⁻¹ atm⁻¹), and \( pK' \) is the dissociation constant of the CO₂–HCO₃⁻ system.

Calculated for the pH₁ prevailing at the beginning of the recovery phase, \( β_{CO_2} \) was 4–8 mM (pH unit)⁻¹. Observing this, the mean acid extrusion rate in bicarbonate Ringer solution was 31 ± 3 mm h⁻¹ (49 measurements from 7 experiments), significantly higher than the rate measured in the same cells in Hepes Ringer solution (26 ± 2 mm h⁻¹, \( P < 0.05 \) on a paired \( t \) test).

The extra buffering power of CO₂–HCO₃⁻ should be fully taken into account only if the hydration–dehydration rate is fast compared with acid extrusion. It seems justified to make this approximation here in spite of the absence of carbonic anhydrase, as the time scale of pH₁ recovery was rather long, on the order of 10 min (Fig. 5).

**Effect of DIDS**

Further evidence for the presence of bicarbonate-dependent regulation is provided by experiments with DIDS, one of

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**Figure 5. Dependence of the pH₁ recovery rate on the presence of CO₂–HCO₃⁻**

The recovery from an acid load produced by 20 mM propionate was first recorded in bicarbonate-free Hepes Ringer solution, and then in bicarbonate Ringer solution (2% CO₂–13 mM HCO₃⁻). Trace 2 was recorded from a small piece of retina, and trace 1 from an individual rod. The sampling interval was 15 s.
the stilbene derivatives commonly used to inhibit anion transport mechanisms. Addition of 100 μM DIDS to CO$_2$-HCO$_3^-$-containing Ringer solution did not as such produce clear changes in steady-state pH$_i$ on a time scale of 10 min (Fig. 6). However, the acid extrusion rate after experimental acidifications was significantly ($P < 0.01$, paired $t$ test) lower in the presence of DIDS ($32 \pm 3$ mm h$^{-1}$; 44 measurements from 6 experiments) than in control bicarbonate Ringer solution ($39 \pm 2$ mm h$^{-1}$).

**Regulation of pH$_i$ in low-Cl$^-$ solution**

Two common bicarbonate-dependent mechanisms for pH$_i$ regulation involve exchange of HCO$_3^-$ for Cl$^-$ (Na$^+$-coupled and Na$^+$-independent HCO$_3^-$-Cl$^-$ exchangers). If such

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**Figure 6. Effect of DIDS on the recovery of pH$_i$ from an acid load**

The rods were first acidified with a 20 mM NH$_4$Cl prepulse in control bicarbonate Ringer solution (2% CO$_2$-13 mM HCO$_3^-$), and then in the same solution containing 100 μM DIDS. The sampling interval was 15 s.

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**Figure 7. Dependence of pH$_i$ on the extracellular Cl$^-$ concentration**

The rods were first acidified with 20 mM propionate in control bicarbonate Ringer solution (2% CO$_2$-13 mM HCO$_3^-$), and then in low-Cl$^-$ solution in which 95 mM NaCl had been replaced with sodium glucuronate. The trace represents the mean of five inner segments from the same experiment. The sampling interval was 15 s.
exchange is present, lowering [Cl\(^-\)] \(_o\) is predicted to drive HCO\(_3^-\) (base) into the cell, as a strongly outward Cl\(^-\) gradient is established. Indeed, replacing 95 mM Cl\(^-\) by glucuronate in bicarbonate Ringer solution caused a clear although transient increase in pH \(_i\) (Fig. 7). Yet the acid extrusion rate after acidification with propionate or NH\(_4\)Cl pre pulses (40 mM h\(^{-1}\)) was not significantly reduced in low Cl\(^-\) solution compared with standard bicarbonate Ringer solution (42 mM h\(^{-1}\)). The latter observation, however, is no strong indication against a HCO\(_3^-\)-Cl\(^-\) exchanger, as its affinity for Cl\(^-\) ions may be sufficient for activation even at low Cl\(^-\) concentrations. It is worth noting that the presence of Na\(^+\)-coupled and Na\(^+\)-independent HCO\(_3^-\)-Cl\(^-\) exchangers simultaneously working in opposite directions could explain the modest net change in acid extrusion when bicarbonate is added.

**DISCUSSION**

**Steady-state pH**

The mean steady-state pHg was 7.09 when measured in nominally HCO\(_3^-\)-free medium (pHg = 7.5). In comparison with values from other vertebrate neurones this appears as typical, but the variation in the literature is wide and may in fact tell more about experimental procedures than about fixed biological properties. For example, in acutely isolated rat hippocampal neurones, Schwiening & Boron (1994) found a mean pHg of 6.81 in nominally HCO\(_3^-\)-free solution (at pHg = 7.4), rising to ca pH 7.1 upon addition of 25 mM CO\(_2\)-HCO\(_3^-\), while Raley-Susan, Sapolsky & Kopito (1993) found a mean steady-state pHg of 7.76 (in the presence of bicarbonate). In cultured neurones, pHg values in nominally bicarbonate-free Ringer solution range from 7.00 (Ou-yang, Mellergård & Siesjö, 1993) to 7.49 (Pocock & Richards, 1992). It is worth noting that isolation of retinal rods is a much faster and gentler process than dissociation of brain cells, so in this respect experimental conditions for our cells might be closer to physiological conditions. We know that a majority of morphologically healthy-looking salamander rods show reasonably normal photocurrent responses after isolation procedures similar to those used here.

Preparation-related differences are highlighted when our value of pH 7.09 is compared with the mean value of pH 6.68 obtained for rods in the isolated, superfused frog retina by Kalamkarov, Pogozheva, Shevchenko, Koskelainen, Hemilä & Donner (1996) (bicarbonate-free solution, pHg = 7.5). These authors recorded a mass pHg signal from the rod layer of intact, fully bleached retinas. Disregarding the possibility of a drastic species difference, there are two factors of apparent relevance to pHg. First, the total bleach in the experiments of Kalamkarov et al.(1996) could have caused a large displacement of pHg at the outset, which might in turn have affected other mechanisms maintaining ion gradients, etc. Also, the rods may have been permanently hyper-polarized. Second, their rods remained embedded in the retinal tissue, exposed to acid fluxes from neighbouring photoreceptors and other retinal cells.

**Buffering power**

The intrinsic intracellular buffering power (β) is, in effect, the slope of the titration curve of all ionizable intracellular compounds with a pK\(_a\) value in the physiological pH range. The curve appeared linear in the pHg range 6.6-8.0, implying that there was no significant dependence of β, on pHg. The mean value, 24 mM (pH unit)\(^{-1}\), is not remarkably high, a somewhat unexpected result in view of some a priori considerations. For example, calculations cited by Liebman et al. (1984) have suggested a value of 50 mM (pH unit)\(^{-1}\). Values measured in vertebrate neurones range from 10-7 mM (pH unit)\(^{-1}\) in rat Purkinje cells (Gaillard & Dupont, 1990) to 27 mM (pH unit)\(^{-1}\) in rat cortical neurones (Katsura, Mellergård, Theander, Ou-yang & Siesjö, 1993).

**The effect of light stimulation**

Light-evoked changes in the extracellular pH of the retina have been studied previously using ion-sensitive electrodes. In the isolated frog retina (Borgula et al. 1989) and in the cat retina in vivo (Yamamoto et al. 1992), illumination induced an alkalinization, which was smaller in the subretinal space surrounding the photoreceptors than in the more proximal layers of the retina. In toad retina the response was biphasic; after 90 s of continuous illumination, the initial acidification turned into an alkalinization, which continued after the light stimulus was removed (Oakley & Wen, 1989). Blocking synaptic transmission with aspartate decreased the acidification without affecting the alkalinization, suggesting that the latter originated in the photoreceptors. This pH rise could have resulted from decreased energy metabolism as the circulating current was suppressed by illumination.

We never saw any clear intracellular light-induced alkalinizations. On the contrary, increasing the frequency of light stimulation often evoked a slow acidification, which stopped when the frequency was decreased. This agrees with the notion that the rods were continuously saturated, so there were no light-induced current changes. In such a situation, illumination would mainly act to increase acid production: first, by H\(^+\) release from cGMP hydrolysis and, second, by the increased metabolism required to restore ATP, GTP and cGMP. Energy metabolism gives rise to lactic acid from glycolysis in the inner and outer segments (Winkler, 1981; Hsu & Molday, 1994) and CO\(_2\) from aerobic metabolism in the inner segment. In basic agreement with this scheme, Haugh-Scheidt, Griff & Linsenmeier (1995) found that retinal oxygen utilization was decreased by illumination in normal Na\(^+\), but was increased by illumination in low Na\(^+\), where changes in the light-sensitive current and the Na\(^+\)-K\(^+\) pump would be damped. It must be admitted, however, that transient changes in acid–base balance would be more difficult to detect in the well-buffered cytoplasm of the rod than in the poorly buffered extracellular solution.
Mechanisms of pH$_i$ regulation

Rods recovered from imposed acidifications, even in the nominal absence of HCO$_3^-$ ions. The mechanism showed obligatory dependence on extracellular Na$^+$ ions, partial inhibition by amiloride and retardation by low pH$_o$. All these features are consistent with the operational definition of the Na$^+$–H$^+$ exchanger.

It is also clear that at least one HCO$_3^-$-dependent mechanism contributes to pH$_i$ regulation. Adding CO$_2$–HCO$_3^-$ to nominally bicarbonate-free medium raised pH$_i$ and increased the acid extrusion rate, albeit modestly. The anion transport inhibitor DIDS reduced the extrusion rate by roughly the same amount (ca 20%) as it had been increased by CO$_2$–HCO$_3^-$. The fact that lowering extracellular Cl$^-$ caused an alkalinization specifically implicates Na$^+$-independent HCO$_3^-$/Cl$^-$ exchange. On the other hand, the accelerated acid extrusion, and its persistence in low [Cl$^-$], is consistent with the Na$^+$-dependent type of exchanger. Thus there are indications that both types of exchange contribute to pH$_i$ control.

Correlation with changes in the light-sensitive current

Changes in the light-sensitive current of isolated salamander rods under experimental treatments similar to some of those used here have been described by Koskelainen et al. (1994). The purpose of their experiments was to characterize pH$_i$ regulating mechanisms, and effects were interpreted in terms of pH$_i$ changes. The basis for interpretation was that, in a certain range, current decreases correlate with acidifications and vice versa (Liebman et al. 1984). With respect to Na$^+$–H$^+$ exchange, the electrophysiology and pH$_i$ measurements are fully consistent. Rod photocurrent was invariably depressed or abolished by amiloride. Unfortunately, Na$^+$ substitution could not be used as a tool in the physiological experiments, as it suppresses the current for reasons unrelated to pH (Hodgkin, McNaughton & Nunn, 1985).

The effects of CO$_2$–HCO$_3^-$ buffer on phototransduction have been described in several studies. Lamb et al. (1981) and Lamb (1984) found that response kinetics in single toad rods was faster in bicarbonate-containing solutions than in pure Heps buffer. In the isolated toad and frog retina, bicarbonate has been found to increase the saturating response amplitude and the concentration of cGMP of rods (Meyertholen et al. 1986; Donner et al. 1990b). These effects are consistent (see Liebman et al. 1984) with the alkalinization we observe when introducing CO$_2$–HCO$_3^-$, and the higher steady-state pH$_i$ of rods continuously kept in bicarbonate-containing perfusates.

On the whole, however, in the electrophysiological studies, bicarbonate-dependent regulation appeared to be more essential than it did in our present pH$_i$ study. For example, DIDS when applied to the inner segment of a rod was often able to abolish completely the light-sensitive current over a time scale of 10 min to 1 h (Koskelainen et al. 1994). This seems to contrast with the rather modest acid extrusion component (ca 20%) contributed by bicarbonate, and inhibited by DIDS, in our experiments. One possibility is that bicarbonate-dependent mechanisms could be relatively upregulated if rods are incubated for longer periods in bicarbonate-containing solution. In the present experiments, all initial preparative procedures were done in nominally bicarbonate-free Ringer solution. The balance between Na$^+$–H$^+$ exchange and bicarbonate-dependent mechanisms might be different in vivo with a continuously high concentration of CO$_2$–HCO$_3^-$. For example, the natural arterial concentration in salamanders is 24 mm (Toews & Boutilier, 1986).


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