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pH Changes in Frog Rods upon Manipulation of Putative pH-regulating Transport Mechanisms

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Rod intracellular pH (pHi) in the intact frog retina was measured fluorometrically with the dye 2,7'-bis(2-carboxyethyl)-5(and-6)-carboxyfluorescein under treatments chosen to affect putative pH-regulating transport mechanisms in the plasma membrane. The purpose was to relate possible pH changes to previously reported effects on photoresponses. In nominally bicarbonate-free Ringer, application of amiloride (1 mM) or substitution of 95 mM external Na⁺ by K⁺ or choline triggered monotonic but reversible acidifications, consistent with inhibition of Na⁺/H⁺ exchange. Bicarbonate-dependent mechanisms were characterized as follows: (1) Replacing half of a 12 mM phosphate buffer by bicarbonate caused a sustained rise of pHi; (2) Subsequent application of the anion transport inhibitor 4,4'-disothiocyanato stilbene-2,2'-disulphonic acid (DIDS, 0.2 mM) set off a slow acidification. (3) Substitution of external Cl⁻ by gluconate (95 mM) caused a rapid pHi rise both in normal Na⁺ and low-Na⁺ perfusion. (4) This effect was inhibited by DIDS. The results support a consistent explanation of parallel electrophysiological experiments on the assumption that intracellular acidifications reduce and alkalinations (in a certain range) augments photoresponses. It is concluded that both Na⁺/H⁺ exchange and bicarbonate transport control rod pHi, modulating the light-sensitive current. Part of the bicarbonate transport is by Na⁺-independent HCO₃⁻/Cl⁻ exchange, but a further Na⁺-coupled bicarbonate import mechanism is implicated. Copyright © 1996 Published by Elsevier Science Ltd.

pH regulation Phototransduction Ion transport

INTRODUCTION

Phototransduction in retinal rods is suppressed by treatments thought to acidify cells (Stillman et al., 1972; Liebman et al., 1984; Meyertholen et al., 1986) and photoresponses are modulated by manipulations known to affect pH-regulating ion transport mechanisms in many cell types (Donner et al., 1990; Katz & Oakley, 1990; Koskelainen et al., 1994). The apparent implication is that rods possess transport mechanisms that can modulate phototransduction by controlling intracellular pH (pHi).

We now report evidence based on actual pHi measurements for the presence of such mechanisms in rods. The signal we record is the maximum fluorescence of 2',7'-bis(2-carboxyethyl)-5(and-6)-carboxyfluorescein (BCECF) loaded rods in the isolated, superfused frog retina. The experimental treatments were chosen to parallel those used in electrophysiological studies on ERG mass receptor photoresponses from the intact retina (Donner et al., 1990; Koskelainen et al., 1993) or the light-sensitive current of isolated rods (Koskelainen et al., 1994). Focusing on the roles of Na⁺/H⁺ exchange and bicarbonate transport (HCO₃⁻/Cl⁻ exchange and Na⁺-coupled HCO₃⁻ transport), the experiments rely on a commonly applied typology of transport mechanisms derived from a broad spectrum of animal cells.

We find that both Na⁺/H⁺ exchange and bicarbonate transport are important for pHi control. Part of the latter is by Na⁺-independent HCO₃⁻/Cl⁻ exchange, but a Na⁺-coupled mechanism is also implicated. The results support a consistent explanation of previously described effects on photoresponses in terms of changes in pHi.

METHODS

Measurement of intracellular pH with BCECF

Intracellular pH (pHi) in the rod layer of the isolated frog retina was measured fluorometrically by means of the intracellular indicator dye BCECF. The fluorescent emission of the dye (peaking at 525 nm) in response to excitation with 490 nm light depends strongly on pH. Emission changes may, however, include components unrelated to pH, e.g. dye loss by bleaching or leakage out of the cell. Such components can be monitored and...
factored out by measuring fluorescence also with excitation at 440 nm, where emission is independent of pH. The ratio of fluorescence intensities evoked by 490 and 440 nm ($I_{490}/I_{440}$) is a fairly pure measure of pH. Thus, every pH value is based on two fluorescence readings close in time. In our experiments, excitation was switched between 490 and 440 nm at 1 or 2 sec intervals. Fuller descriptions of the method can be found for example in Paradiso et al. (1987), DeVries and Schwartz (1989) and Takahashi et al. (1993).

**Preparation and dye loading**

*Loading.* Retinas isolated from frogs (*Rana temporaria*) that had been quickly decapitated and double-pithed were flat-mounted photoreceptor side up on a filter paper placed on a piece of glass. Rods were loaded in full room light by incubating the photoreceptor side of the retina with the neutral (membrane-permeant) acetoxyethyl-ester form (BCECF AM) of the indicator dye. In this form, it freely enters cells, where it is hydrolysed by intracellular esterases to negatively charged (non-permeant) BCECF, which stays trapped inside the cell. A 10 µM solution of BCECF AM (prepared by adding 10 mM stock solution in dimethyl sulfoxide to phosphate Ringer at pH 7.5) was applied, washed off and applied again at 1 min intervals for 5 min at room temperature. Then the retina was washed twice in phosphate Ringer. The purpose was to ensure sufficient loading of rods while minimizing dye penetration deeper into the retina (see below).

*Control of dye localization.* Dye localization was checked in 10 µm slices of retinas which had been frozen and sectioned on a microtome table immediately after loading [see Zak et al. (1974)]. The technique provides a good resolution of retinal layers. In the first five slices, the only major proteins are rhodopsin, G-protein and arrestin [see Donner et al. (1990)], showing that there is no significant contamination from structures other than rod outer segments. In the following slices, rhodopsin drops below 5% of all protein and many inner-segment proteins appear, together with succinate dehydrogenase activity, a marker for mitochondrial (inner-segment) structures (Zak et al., 1974). The identifications are consistent with histological data on the length of rod outer segments in *R. temporaria*, for which Hemilä and Reuter (1981) found a mean value of 43 µm. (Note that the pigment epithelium had been peeled off and Müller cell processes extend only to the level of the inner segments.)

In retinas frozen immediately after the 5 min loading protocol, about 80% of all dye was confined to the first three slices, corresponding to the outer segment layer. Some of this dye may have been located in cones, but in view of the at least 50-fold rod/cone volume ratio in the layer of photoreceptor outer segments [see for example Saxén (1954)], the cone contribution to the measured fluorescence signal cannot have been significant. By the start of the pH measurements, the dye originally captured in a rod outer segment would have diffused to more or less uniform concentration throughout the cell, so the signal we measure is a whole-rod signal, not differentiating between inner and outer segments. Any remaining extracellular dye would have been washed off by perfusion.

Before starting the main measurements, we recorded the emission spectrum of the retina to check that the rods gave a sufficiently strong fluorescence. Controls showed that BCECF accumulation was similar in dark-adapted and bleached retinas. Recordings of the aspartate-isolated mass rod (ERG) potential across dark-adapted retinas indicated that loading by BCECF did not significantly change photoresponses. The saturated response amplitude decreased by only 20% after 10 min perfusion with BCECF AM Ringer, and sensitivity and response kinetics were similar to those recorded in untreated retinas.

*Recording and calibration of pH.*

*Recording and perfusion.* The piece of glass carrying the flat-mounted retina was positioned vertically at 45 deg angle to the incident beam in a 3 ml quartz cuvette in the spectrofluorometer (Hitachi). Fluorescence (at 525 nm, 2 nm bandwidth) was measured at 90 deg angle to the excitation light. The inlet tube for perfusion ended at the bottom of the cuvette immediately below the retina; the outlet was near the upper corner. Perfusion was gravity-driven at 1.2 ml min⁻¹. Effectively, the solution bathing the retina was changed in 0.5–1 min.

*Calibration.* The relation between fluorescence ratio and pH was calibrated in conventional manner by adding 10–20 µM nigericin to the perfusate at the end of the experiments. Nigericin is a H⁺/K⁺ exchanger used here to make the cell membrane permeable to H⁺. When potassium is equilibrated across the membrane (with 120 mM KCl in the external solution), pH will be almost equal to pHᵢ. The calibration is done by recording the $I_{490}/I_{440}$ fluorescence ratio while the retina is perfused with Ringers adjusted to different pH values [cf Nuccitelli & Deamer (1982)]. The ratio depended approximately linearly on pH in the range 6.2–7.5. Calibration was not performed in every single retina studied, as the calibration function remained relatively constant between preparations.

*Solutions.* The Ringer solutions contained (in mM): NaCl 95, KCl 3, CaCl₂ 0.9, MgCl₂ 0.5, glucose 10, and pH buffers 12. The buffer was 12 mM sodium phosphate (phosphate Ringer) or 12 mM HEPES (HEPES Ringer) or either of these with half (6 mM) of the buffer replaced by bicarbonate (bicarbonate Ringer). pH was adjusted to 7.5 by adding NaOH or HCl. When used together with other buffers, bicarbonate at this low concentration and high pH requires no bubbling, as CO₂ escapes very slowly. This conclusion is based on measurements of pH changes over 6 hr in Ringers buffered with either 12 mM bicarbonate or 6 mM HEPES +6 mM bicarbonate and kept in open beakers, as described by Koskelainen et al. (1993).

*Experimental treatments.* The experimental treatments were chosen to closely parallel those previously used in
electrophysiological experiments. The primary targets are a few acid–base transport mechanisms commonly identified in animal cells. The Na⁺/H⁺ exchanger is a near-ubiquitous feature of the plasma membrane of excitatory cells [see Piwnica-Worms et al. (1988)]. It is retarded or stopped if external sodium is replaced by, for example, potassium or choline (Aronson, 1985; Mahnessmith & Aronson, 1985), and at least one subclass is inhibited by amiloride [see Benos (1988)]. Of two main types of bicarbonate/chloride exchangers, one is coupled to the driving force of sodium, while the other is sodium-independent (see, for example, Thomas, 1984; Madshus & Olsnes, 1987; Boron & Knakal, 1992). A further Na⁺-coupled bicarbonate transport mechanism that has been found in retinal pigment epithelium and glial cells, but not to date in neurons, is the electrogenic Na⁺/HCO₃⁻ coporter (Boron & Boulpaep, 1983; Hughes et al., 1989; laCour, 1989; Newman & Aston, 1991). All these bicarbonate transport mechanisms are inhibited by stilbene derivatives, e.g. 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) (Cabantchick et al., 1978; Roos & Boron, 1981; Hughes et al., 1989; laCour, 1989).

The chemicals employed to affect acid–base transport were: (1) amiloride (1 mM); (2) DIDS (0.2 or 0.5 mM); (3) nigericin (10 or 20 μM) (all from Sigma). BCECF AM was obtained from Molecular Probes (Oregon, U.S.A.).

Remarks on the preparation

We recorded BCECF-fluorescence as a mass signal from the rod layer of intact retinas where the visual pigment had been bleached, a technique that has not to our knowledge been used previously. There are two points that merit comment: (1) the relation to measurements in isolated rods; (2) the consequences of a full rhodopsin bleach with respect to pHᵢ.

The whole-retina preparation for studying rod pHᵢ.

The use of intact retinas entails sacrifices, for example, in spatial and temporal resolution compared with single-cell recording, but on the other hand also offers important advantages. The rods are at no stage disrupted and remain in a reasonably normal milieu, including flow of metabolic acid from neighbouring photoreceptors and from the inner retina [see Borgula et al. (1989); Oakley & Wen (1989); Donner et al. (1990)]. They also tend to remain in a better condition, in connection with photocurrent recordings on dissociated cells we have often observed that rods attached to small pieces of retina, or even bunches of a few connected rods, continue to look morphologically healthy and respond to light stimuli when isolated cells already start to burst or show clear signs of decay. Further, the signal recorded is the average from thousands of rods and thus has a fairly high signal-to-noise ratio. The advantages and disadvantages are analogous to those of whole-retina ERG compared with single-cell recordings of photocurrent or photovoltage. The obvious problem of isolating a rod signal, that is, eliminating admixtures from other retinal structures, can be managed reasonably well by appropriate procedures for dye loading (see above).

Fluorometry in photosensitive cells. A general problem with fluorometrical techniques in photoreceptor cells is that, by definition, they involve the use of considerable amounts of light. Thus, the measurement itself alters the state of the cells. Here we chose to bleach the rhodopsin at the outset to ensure invariant conditions throughout the experiments. The bleach and inactivation of phototransduction may well alter the level of acid turnover and displace steady-state pHᵢ compared with photosensitive rods, but there are no grounds for assuming that basic (qualitative) properties of ion transporters in the plasma membrane should have been changed.

RESULTS

Steady-state pHᵢ

Steady-state pHᵢ measured at the start of experiments in nominally bicarbonate-free Ringer at pHᵢ = 7.5, averaged 6.68 ± 0.05 (SEM, 11 retinas where absolute pHᵢ was carefully calibrated). The value is considerably lower than typically found in vertebrate neurones, and possible reasons are considered in the Discussion. When judging the relative acidity, one relevant factor is the (nominal) absence of CO₂/HCO₃⁻. In four retinas that had been isolated and incubated in bicarbonate Ringer from the outset, pHᵢ averaged 6.90 ± 0.15. The effect of bicarbonate is further demonstrated in some of the experiments below (see Fig. 3).

Na⁺/H⁺ exchange

Effects of amiloride. In electrophysiological experiments, amiloride has been found to depress rod photoresponses both when applied to the intact retina [Katz & Oakley (1990); our unpublished observations on the ERG] and when applied to either the inner or outer segment of isolated rods (Koskelainen et al., 1994). Effects on photoresponses could, however, to an
unknown degree be due to other documented actions of the drug, such as blocking of Na⁺/Ca²⁺, K⁺ exchange or (some) sodium channels (Benos, 1988; Nicol et al., 1987). In experiments on pH₃, amiloride can be considered as a fairly specific inhibitor of Na⁺/H⁺ exchange. It is not known to interfere with bicarbonate transport.

Application of 1 mM-amiloride in nominally bicarbonate-free Ringer always set off a steady acidification (Fig. 1, showing results typical of nine similar experiments). Although substantial, it was always much slower than that following Na⁺ substitution (see Fig. 2). The acidification was always halted when amiloride was washed off, but the degree of reversibility varied. If amiloride was on for a shorter period only, so pH₃ had not changed by >0.1 units before washout, the effect was completely reversible. If pH₃ was allowed to drop by >0.3 units before washout, recovery was always incomplete (cf the results with sodium substitution below).

Sodium substitution. Substitution of external Na⁺ by K⁺ or other cations is a more powerful way of stopping (or even reversing) H⁺ extrusion by Na⁺/H⁺ exchange, but has two major drawbacks:

1. It will affect all Na⁺-dependent transport mechanisms, including Na⁺-coupled bicarbonate transport.
2. Parallel experiments on photoresponses are not possible, because removal of Na⁺ leads to a rapid loss of the light-sensitive current (Hodgkin et al., 1985).

We used two different cations, potassium and choline, to substitute for sodium in different experiments. The purpose was to compare situations where the changes in membrane potential were expected to go in opposite directions. The experiments were done in nominally bicarbonate-free phosphate Ringer to minimize contributions from HCO₃⁻ transport. Figure 2 shows results from two experiments, one with potassium (A) and one with choline (B). In both situations, strong acidifications were observed, and the similarity of the results indicates that changes in membrane potential play at most a secondary role.

In Fig. 2(A), 95 mM NaCl in the control solution was replaced by equimolar KCl at 3 min (leaving only the Na⁺ of the phosphate buffer). This set off a monotonic acidification. The mean (± SEM) initial acidification rate in eight similar experiments was 0.024 ± 0.008 pH units min⁻¹. After 10 min, when pH₃ had dropped by 0.08 units, perfusion was returned to control and pH₃ recovered. The acidification was always halted by return to normal [Na⁺], but the degree of recovery varied (cf the amiloride experiments above). If sodium had been substituted for a shorter period only and pH₃ had changed by <0.15 units, the effect was completely reversible. It was completely irreversible if pH₃ was allowed to drop by >0.3 units before return to normal sodium. Strong acidosis or some other effect of Na⁺-depletion appears to inactivate the regulation mechanisms.

In Fig. 2(B), 95 mM Na⁺ was substituted by equimolar choline. Two experiments of this kind gave a mean initial acidification rate of 0.025 pH units min⁻¹. Thus there was no clear difference compared with the Na⁺→K⁺ substitution.

Bicarbonate transport

Donner et al. (1990) found that replacing 6 mM of a 12 mM HEPES or phosphate buffer by bicarbonate in the Ringer superfusing an isolated retina led to:

1. A sustained doubling of total [cGMP] in rods.
2. A sustained growth of the saturated photoresponse of rods by 30% on average.

They hypothesized that the common cause could be a pH₃-rise mediated by mechanisms that transport bicarbonate (HCO₃⁻) into the cells.

In Fig. 3 the retina was first perfused with nominally bicarbonate-free phosphate Ringer. When bicarbonate Ringer (containing 6 mM HCO₃⁻) was introduced, pH₃ started to rise. As long as bicarbonate was left on, pH₃ remained at the elevated level. In eight experiments, the
mean value of the sustained rise was $0.26 \pm 0.04$ pH units.

It should be noted that increasing $[\text{HCO}_3^-]_o$ at constant pH$_o$ is necessarily associated with elevated CO$_2$-levels both outside and inside cells (because CO$_2$ diffuses freely across membranes). The hydration reaction inside the cell (CO$_2$+H$_2$O→H$_2$CO$_3$→H$^+$+HCO$_3^-$) will then liberate hydrogen ions, and this should push pH$_i$ in the acidic direction. Therefore, the alkalinization observed when $[\text{HCO}_3^-]_o$ is increased is strong evidence for the presence of mechanisms that transport bicarbonate into the cell. It may seem surprising that in Fig. 3 there is no trace even of an initial acidification transient from the CO$_2$ increase. However, this may be explained by the relative slowness of CO$_2$ hydration in rods, which apparently lack the enzyme carbonic anhydrase (Musser & Rosen, 1973; Linser & Moscona, 1984). If HCO$_3^-$ import is fast enough, the balance of the hydration/dehydration reaction is shifted to the left before any measurable acidification has occurred.

Figure 3(B) illustrates an experiment where the anion transport inhibitor DIDS (0.2 mM) was applied just after the bicarbonate-induced alkalinization. DIDS set off a monotonic decrease of pH$_i$. Four experiments of this type all gave similar results, consistent with inhibition of the bicarbonate-importing mechanism.

The bicarbonate transport involves HCO$_3^-$/Cl$^-$-exchange

Lowering external Cl$^-$ raises intracellular pH. In suction pipette experiments on isolated rods, decreasing [Cl$^-$], around the outer segment (with [Ca$^{2+}$], carefully kept constant) has been found to increase the photocurrent (Koskelainen et al., 1994). Similarly, decreasing perfusion [Cl$^-$] increased the amplitude of mass rod responses from the isolated retina (our unpublished observations). A possible explanation would be alkalinization mediated by HCO$_3^-$/Cl$^-$ exchange: creating an outward drive for chloride by lowering [Cl$^-$], would accelerate HCO$_3^-$ import and thereby alkalinize cells. The experiments illustrated in Fig. 4 support this idea.

Substitution of 95 mM NaCl by equimolar Na-gluconate
The chloride substitution effect persists in low sodium. At time 0, the retina had been for 3 min in a perfusate where 95 mM NaCl had been replaced by equimolar KCl. 95 mM KCl was in turn replaced by equimolar K-glucocand as shown by the horizontal bar.

Bicarbonate buffer.

FIGURE 5. The chloride substitution effect persists in low sodium. At

time 0, the retina had been for 3 min in a perfusate where 95 mM

NaCl had been replaced by equimolar KCl. 95 mM KCl was in turn

replaced by equimolar K-glucocand as shown by the horizontal bar.

Bicarbonate buffer.

(A) indeed resulted in a strong rise of pH. The effect was

fully reversible, and the chloride-glucocand-chloride
cycle could in fact be repeated several times in

succession without significant decrease of the amplitude

of the pH response. The mean pH rise was 0.15 ± 0.02

pH units (seven retinas).

The Cl− substitution effect is DIDS-sensitive. If the

chloride substitution effect is mediated by HCO3−/Cl−

exchange, it should be suppressed by DIDS. In four

experiments, exemplified by Fig. 4(B), this was found to

be the case. First we checked that the retina gave the

usual alkalinizing response to chloride-glucocand sub-

stitution. Return to normal [Cl−]o set off a descent

towards the original baseline. Then 0.2 mM-DIDS was

added, and when pH had descended to the original level,

chloride was again replaced by glucocand. The alkaliniz-

ing response was always strongly reduced in the presence

of DIDS.

Figure 4(B) brings a further observation of consider-

able importance. The addition of DIDS was always done

in the middle of the relaxation transient after the return to

normal [Cl−]o. In all four experiments, DIDS accelerated

the ongoing acidification. In this phase, however, the

driving force for Cl− should be clearly inward. The

accelerated acidification suggests that DIDS here inhibits

a mechanism that transports HCO3− into the rods

regardless of the driving forces for Cl− or HCO3−.

Such a mechanism would necessarily have to be coupled
to Na+ (see Discussion).

Evidence for Na+-independent HCO3−/Cl− exchange

To test specifically for the presence of the Na+-
independent HCO3−/Cl− exchanger, Cl− substitution

was done also in low-Na+ (containing only 6 mM Na+ from

the buffer). We chose not to remove all Na+, because the

very rapid acidification may drive rods into an

irreversible state very quickly, before the Cl−

substitution can be performed (see the discussion of the

Na+-substitution experiments, Fig. 2).

First 95 mM NaCl was substituted by equimolar KCl, then the KCl was replaced by equimolar K-glucocand. In this situation, where [Na+]o is decreased by more than one order of magnitude, Na+-dependent bicarbonate import should be strongly depressed, and if the alkalinizing response to Cl− removal persists, it indicates the presence of HCO3−/Cl− exchange not coupled to Na+.

In Fig. 5, the record starts in low Na+. To avoid excessive acidification, chloride was switched to glucocand within a few minutes. The resulting alkalinization was in fact similar to that in normal Na+ (mean 0.18 ± 0.05 in three experiments), suggesting that a substantial part of the bicarbonate transport is by Na+-

independent HCO3−/Cl− exchange.

DISCUSSION

Steady-state pH

The mean steady-state pH, 6.68 (in nominally bicarbonate-free perfusion at pHo = 7.5) is untypically low for excitable cells, and might indeed be unrepresentative of functioning rods in vivo (see below). However, values for neurones reported in the literature vary substantially, and details of preparation and experimental conditions apparently strongly affect the results. Schwiening and Boron (1994), for example, report a mean value of 6.81 for isolated rat hippocampal neurones in nominally bicarbonate-free medium (pH0 = 7.4), which rose to c. 7.1 when the HEPES-buffer was replaced by 25 mM bicarbonate, while Raley-

Susman et al. (1993) obtained a mean value of 7.76 in the same type of neurones in 25 mM bicarbonate and

pH0 = 7.4.

At least three factors seem relevant for assessing the significance of our present value. First, the visual pigment was bleached and phototransduction inactivated.

This may have displaced steady-state pH by several different mechanisms. Second, the rods remained embedded in retinal tissue, exposed to acid fluxes from neighbours and other retinal cells. The extracellular pH profile recorded by Oakley and Wen (1989) in the toad retina suggests that pHo is at least 0.1 unit below perfusion pH around the inner–outer segment junction, and 0.2 units at the proximal ends of rods. Third, pH = 6.68 was obtained in the nominal absence of bicarbonate. Introducing 6 mM HCO3− in the perfusate raised pH by 0.26 units on average, and in vivo the concentration of CO2−−HCO3− is much higher. In salamanders, for example, arterial [HCO3−] at room temperature is 20–25 mM (Toews & Boutilier, 1986). For this reason alone, rods in vivo are very likely to have a higher steady-state pH.

Comparison with physiology

Our central result is that the pH changes observed can be consistently correlated with effects on rod
photoresponses observed in analogous electrophysiological experiments. The general rule is that acidification correlates with response reduction, alkalinization with response growth (in a certain range). The correlations concern directions and rates of change and appear as rather robust in relation to possible differences in steady-state pH between the bleached rods studied here and the dark-adapted rods studied electrophysiologically.

1. Amiloride has been found to depress rod photoresponses both when applied selectively to the inner or to the outer segment of isolated rods, and when applied to the intact superfused retina (Koskelainen et al., 1994; Katz & Oakley, 1990; our unpublished observations). Since amiloride is a far from specific agent in electrophysiological experiments, the present pH measurements (Figs 1 and 2) provide crucial evidence for the notion that at least part of its physiological action is due to inhibition of Na⁺/H⁺ exchange.

2. In the intact superfused retina, introduction of 6 mM bicarbonate in previously bicarbonate-free Ringer has been found to cause sustained rises of both the saturated response amplitude and the cGMP level of rods (Donner et al., 1990). This correlates with the sustained rise of pH observed here after the same treatment [Fig. 3(A)].

3. Application of DIDS to the intact superfused retina has been found to completely suppress rod photosensitivity unless pHₗ is high (Donner et al., 1990). In isolated rods, DIDS suppressed photoresponses when applied in bicarbonate Ringer to the inner segment, but not when applied to the outer segment (Koskelainen et al., 1994). The suppressive effects of DIDS developed slowly, on a time scale of tens of minutes to 1 hr. These results correlate well with the slow, steady pHₗ decline observed here after DIDS application in bicarbonate Ringer [Fig. 3(B)].

4. Substitution of chloride by gluconate around the outer segment of isolated rods (Koskelainen et al., 1994) or in the intact perfused retina (our unpublished observations) has been found to boost photoresponses strongly by a DIDS-sensitive mechanism. This correlates with the strong, DIDS-inhibitable pHₗ rise observed here after Cl⁻ substitution (Fig. 4).

On the other hand DIDS in itself, when applied selectively to the outer segment in suction-pipette recordings, increased photoresponses. Koskelainen et al. (1994) attributed this to inhibition of a Na⁺-independent HCO₃⁻/Cl⁻ exchanger that would normally be extruding base (HCO₃⁻) from the cell (as expected if pHₗ is well above thermodynamic equilibrium, and [Cl⁻]; not very far above the equilibrium value). The present evidence for Na⁺-independent HCO₃⁻/Cl⁻ exchangers in rods (Fig. 5) gives some support to this attribution.

**Functions of pH-regulating mechanisms in rods**

**Acid production and extrusion.** In the functioning rod, acid is produced at a high rate by energy metabolism, including glycolysis in both the inner and outer segments (Winkler, 1981, 1986; Hsu & Molday, 1991, 1994), and by cGMP hydrolysis and synthesis in the outer segment [see Liebman et al. (1984)]. Changes in pH affect the two segments differently, but in both, acidosis is expected to lead to loss of light sensitivity [see discussion in Koskelainen et al. (1994)]. Thus one clear function of pH-regulating transport mechanisms is the extrusion of endogenously produced acid. We find evidence for two processes which, being coupled to the Na⁺ gradient, will always (when active) serve this purpose: Na⁺/H⁺ exchange (Figs 1 and 2) and Na⁺-coupled HCO₃⁻ import. The latter is indicated by the fact that application of DIDS in the presence of bicarbonate always caused net acidification [see point (3) above and Fig. 4(B)]. We had no means, however, of differentiating between, for example, a Na⁺-coupled HCO₃⁻/Cl⁻ exchanger and a Na⁺-HCO₃⁻ coporter.

**Na⁺-independent HCO₃⁻/Cl⁻ exchange.** The third pH-regulating mechanism implicated is a Na⁺-independent HCO₃⁻/Cl⁻ exchanger, present at least in the outer segment [see point (4) above]. The outer segment would thus be equipped with acid-extruding Na⁺/H⁺ exchange and (usually) base-extruding HCO₃⁻/Cl⁻ exchange. In the face of both acidifying and alkalinizing transients known to be associated with normal retinal function (Borgula et al., 1989; Oakley & Wen, 1989), pHₗ homeostasis for phototransduction might be optimally served by a combination of (regulated) mechanisms simultaneously working in opposite directions.

**CONCLUSIONS**

The results indicate that rods possess at least three mechanisms for regulation of intracellular pH:

1. Na⁺/H⁺ exchange;
2. Na⁺-coupled bicarbonate import; and
3. HCO₃⁻/Cl⁻ exchange not coupled to the Na⁺ gradient.

Electrophysiological evidence (Koskelainen et al., 1994) suggests that at least (1) and (2) are active in the inner segment, primarily serving protection against long-term metabolic acidification. At least (1) and (3) appear to be active in the outer segment, providing pHₗ homeostasis and, potentially, a novel regulation pathway for phototransduction.

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