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pH Regulation in Frog Cones Studied by Mass Receptor Photoresponses from the Isolated Retina

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Mass cone photoresponses were recorded across the aspartate-treated frog retina under treatments chosen to affect putative pH-regulating mechanisms. The saturated response amplitude (U_{max}) was found to be a monotonically increasing function of perfusion pH in the range 7–8, and thus presumably of intracellular pH (pH_i). Accepting that U_{max} can be used as an index of pH_i changes, two results indicate the importance of bicarbonate transport for preventing intracellular acidification: (1) bicarbonate-buffered (6 mM HCO_3^- + 6 mM HEPES) perfusate increased U_{max} compared with nominally bicarbonate-free perfusate (12 mM HEPES); (2) the anion transport blocker DIDS (0.1 mM) caused a strong decrease in the amplitude of photoresponses. Substitution of 95 mM chloride by gluconate in the perfusing fluid boosted photoresponses indicating that at least part of the bicarbonate transport involves HCO_3^-/Cl^- exchange. Amiloride (2 mM) also caused a decrease of photoresponse amplitude, which suggests that Na^+/H^+ exchange contributes to pH_i regulation. In all these respects, cones behaved similarly to rods. Cones differed from rods (in the intact retina) in that addition of 0.5 mM of the carbonic anhydrase inhibitor acetazolamide reduced (never augmented) photoresponses. The difference is considered in relation to the presence of carbonic anhydrase in cone, as opposed to rod, outer segments.

Cone photoreceptor Retina Phototransduction pH regulation ERG

INTRODUCTION

We have previously characterized pH regulation in amphibian rod photoreceptors both by recording changes in light responses and changes in pH_i (Donner, Hemilä, Kalamkarov, Koskelainen & Shevchenko, 1990; Kalamkarov, Pogozheva, Shevchenko, Donner, Koskelainen & Hemilä, 1993; Koskelainen, Donner, Kalamkarov & Hemilä, 1993). Here we extend the physiological study of pH effects to cones. Several of the cone/rod differences bear on the acid budget of the cells: (1) the cone outer segment (COS) has some 50 times smaller volume [in the frog retina (Saxén, 1954; Liebman & Entine, 1968)] and its disks are contiguous with the plasma membrane; thus diffusion of substances out of the COS should be more efficient than in rods; (2) the dark rate of cGMP turnover, a major acid load in the outer segment (see Liebman, Mueller & Pugh, 1984; Kalamkarov *et al.*, 1993), is likely to be much higher in cones; (3) the COS, in contrast to the rod outer segment (ROS), has been shown to contain the enzyme carbonic anhydrase (CA) (Musser & Rosen, 1973; Sapirstein, 1983; Linser & Moscona, 1984; Nork, McCormick, Chao & Odom, 1990). This enzyme catalyses the hydration of carbon dioxide to carbonic acid, ensuring a fast equilibrium in the CO_2–HCO_3^- buffer system.

The experimental procedures and interpretations are based on our previous experience from rods. Changes in the saturated response amplitude U_{max} are used to monitor the effects of treatments designed to affect pH_i-regulating mechanisms. In rods, the saturated current response grows monotonically (although at least partly transiently) with rising pH_o (Liebman *et al.*, 1984; Donner *et al.*, 1990; Koskelainen *et al.*, 1993). Here we first demonstrate that a monotonic relation between pH and U_{max} also holds for cone photoresponses recorded as ERG potentials across the isolated and perfused frog retina. When synaptic transmission is blocked by aspartate and rods are suppressed by a blue background light, the ERG photovoltage is essentially a measure of the current response of cones. The whole-retina preparation we use has the advantage that the milieu and physiological state of the cells remain reasonably normal. For example, the flux of metabolic acid from the inner retina (see Oakley & Wen, 1989; Borgula, Karwoski & Steinberg, 1989; Donner *et al.*, 1990) remains patent, as opposed to the situation in isolated cells.

We find that pH control in cones like in rods depends decisively on bicarbonate transport plus, apparently, Na^+/H^+ exchange. A major difference compared with

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rods is that inhibition of retinal CA, which boosts rod photoresponses, reduces cone photoresponses. We interpret this as an expression of a different and possibly even more crucial role of bicarbonate for pH_i control in cones.

MATERIALS AND METHODS

Materials and recording

Cone photoresponses were recorded as ERG mass receptor potentials from the isolated retina of the frog (*Rana temporaria*) where synaptic transmission had been blocked by 2 mM sodium aspartate. The photovoltage across the aspartate-treated retina can (with due discernment) be regarded as the extracellular ohmic voltage generated by the current response of photoreceptors. The relation between the ERG signal and the current response of photoreceptors is critically discussed by Donner and Hemilä (1985) and Donner, Hemilä and Koskelainen (1992).

The frogs were caught in the wild in southern Finland at the end of October and maintained in basins at 4°C without feeding. Before an experiment, a frog was gradually warmed to *c.* 15°C over 18 hr in complete darkness. It was quickly decapitated and double-pithed, the eyes were excised, and the retina of one eye was isolated in cool Ringer solution under dim red light. The retina was mounted in a specimen holder where the photoreceptor (upper) side was perfused and the vitreal side rested on a plexiglass hemisphere (Donner, Hemilä & Koskelainen, 1988). In this geometry, all exchange of substances takes place on the perfused receptor side. Photoresponses were d.c.-recorded as a transretinal voltage with Ag/AgCl electrodes, digitized and stored on disk. When only response amplitudes were measured, eight-pole analogue filtering with cut-off at 20 Hz and a sampling rate of 100 Hz with two-point smoothing was used. When precise response kinetics were recorded, the cut-off of the filter was raised to 100 Hz and the sampling rate to 200 Hz. In fitting model response waveforms [according to equation (2)], $t = 0$ was taken at the middle of the flash and correction was made for the delay introduced by the analogue filtering. The experiments were done at 12°C.

In principle, our measure of cone function here is the amplitude of saturated photoresponses (U_{max}), which reflects the size of the circulating light-sensitive current. Since response amplitude saturates asymptotically with rising stimulus intensity [as described by a Michaelis–Menten relation, see equation (1) and Fig. 1(B)] and we preferred not to use extremely strong flashes, in practice we recorded responses to *nearly* saturating flashes interleaved with responses to dim (essentially linear-range) flashes. The dim-flash responses served to control that sensitivity did not change so as to make our "near-saturating" intensity inadequate. It also served to monitor that dim-flash and saturated response amplitudes did not change in parallel, which effectively rules out the possibility that changes in the recorded ERG photovoltage could be trivially due to changes in extracellular resistance. In the following, we shall denote the amplitude of the near-saturated responses by U_{max} (in fact within 95% of the fully saturated amplitude). Now and then responses to a more complete set of flash intensities were recorded in order to assess the shape of the stimulus–response function.

The two channels of the optical system were independently controlled with interference filters (Schott IL) neutral density filters and wedges to produce stimuli and backgrounds of desired wavelengths and intensities. Stimuli were 30 or 50 msec flashes of 642 nm light. Throughout the experiments, a 430 nm background light was present, sufficiently strong to suppress rods almost completely (see Fig. 1). It also kept the cones in a constant state of moderate light-adaptation.

Solutions and perfusion

The Ringer solutions contained (mM): NaCl, 95; KCl, 3; $CaCl_2$, 0.9; $MgCl_2$, 0.5; glucose, 10; pH buffers, 12. 5% Leibovitz culture medium L-15 (Sigma) was added to the Ringers to improve the viability of the retina. pH was adjusted (by adding NaOH or HCl) to 7.5 unless otherwise noted. The buffer was either 12 mM sodium–HEPES buffer (*HEPES Ringer*), or 6 mM sodium bicarbonate plus 6 mM sodium–HEPES (*bicarbonate Ringer*). When used together with other buffers, bicarbonate at such low concentration and high pH requires no CO_2 bubbling, as CO_2 escapes very slowly. This was confirmed by continuous pH measurements in two Ringer solutions (initial pH 7.5) stored in open beakers: one a test solution buffered with 12 mM bicarbonate alone, the other our standard 6 mM bicarbonate + 6 mM HEPES buffer. Small alkalinizations were observed, amounting to 0.1 pH units in the bicarbonate–HEPES solution after 6 hr. This might in principle, towards the end of long experiments, contribute to "bicarbonate effects". However, the bicarbonate-induced response growth was at least equally prominent at the beginning of experiments (Fig. 3) and anyway far too large to be explained even by a full 0.1 unit alkalinization (cf. Figs 2 and 3). As regards the concentration of bicarbonate, on the other hand, the relative decrease indicated by such small pH changes (the 12 mM bicarbonate solution without other buffers was alkalinized from 7.5 to 7.8 over 6 hr) is obviously negligible. In our experiments (usually lasting no more than 3 hr), CO_2 escaping from our 6 mM HEPES + 6 mM bicarbonate Ringers was clearly not a significant source of error.

Gluconate binds calcium (Skibsted & Kilde, 1972). Therefore, in experiments where 95 mM NaCl was substituted by sodium-gluconate, 2.37 mM $CaCl_2$ was added to keep free [Ca^{2+}] constant. The required addition was calibrated by titration in bicarbonate Ringer at pH = 7.5 while free calcium was monitored with a Ca^{2+}-electrode (Orion Research Corporation model 93-20).

To inhibit HCO_3^- transport we used 0.1 mM 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) and to inhibit Na^+/H^+ exchange 2 mM amiloride (Sigma). To inhibit CA, we used acetazolamide (0.5 mM), which is known to permeate easily into cells

(Maren, 1967). In the gravity-driven perfusion system, one solution replaced another in about half a minute after a switch. This delay has been observed in the figures: switch times indicate when the new solution is estimated to reach the retina.

RESULTS

Cone ERG photoresponses

The aspartate-isolated cone ERG recorded across the perfused frog retina (see Hood & Hock, 1973) has

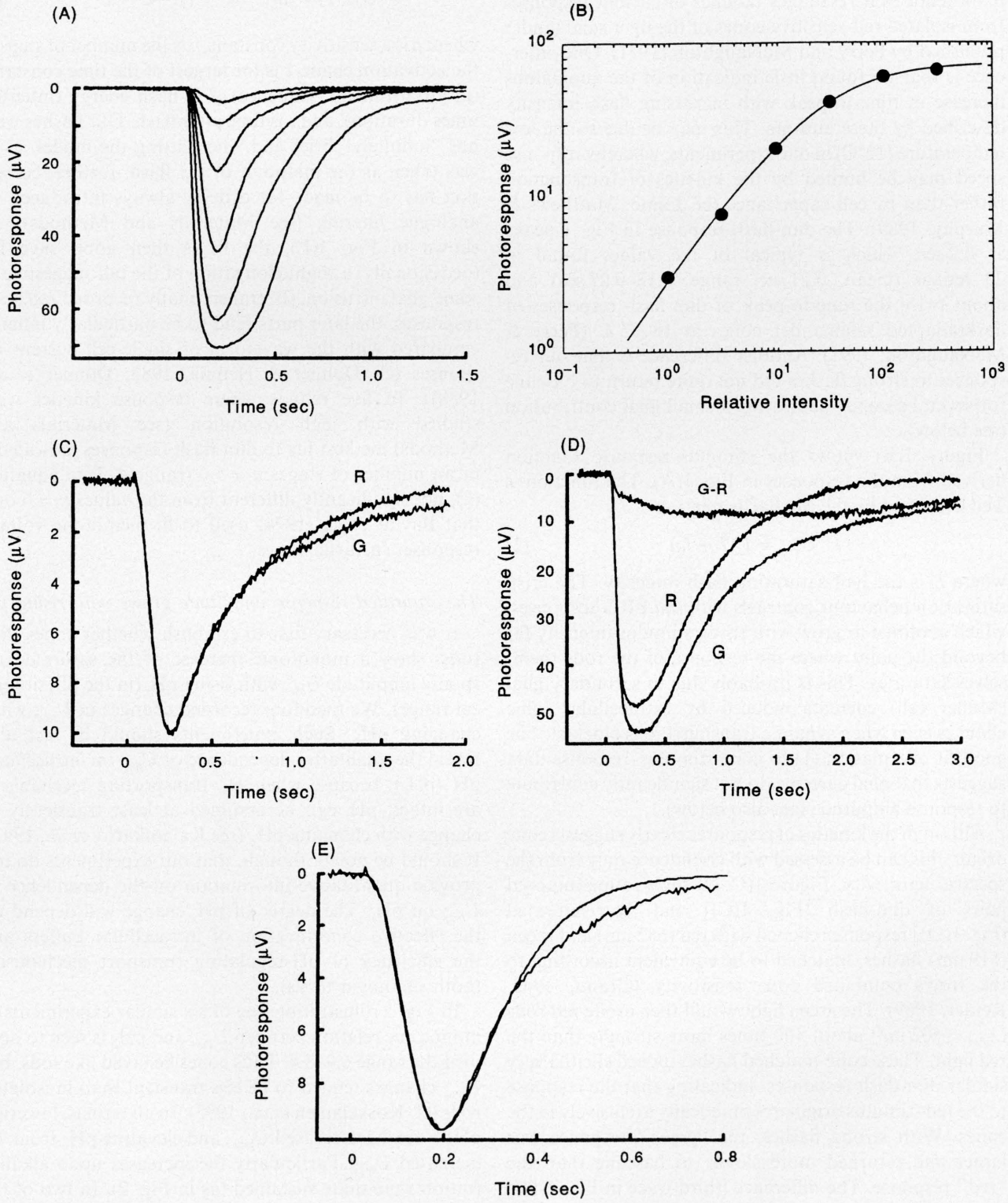

FIGURE 1. Characteristics of the aspartate-isolated cone ERG recorded across the frog retina. Temperature *c.* 12°C. (A) Response family to 30 msec flashes increasing in 0.5 log unit steps. Each trace is an average of four responses. (B) Stimulus–response function generated from (A). The curve is a Michaelis function [equation (1), $U_{max} = 73.7\ \mu V$, $\log I_0 = 1.38$]. (C) A pair of responses to dim cone-matched flashes of "red" (R, 642 nm) and "green" (G, 519 nm) light. (D) A pair of responses to near-saturating, cone-matched R and G flashes. The third trace is the difference of the responses (G − R). (E) The dim-flash response (scaled to unity) fitted with the "independent activation" model [equation (2), $n = 7$, $\tau = 0.1$ sec]. Average of 14 responses.

not been used to full advantage previously, so we first describe this convenient signal. Figure 1(A) shows a typical response family elicited by 642 nm flashes at 0.5 log unit intensity intervals (i.e. covering a 2.5 log unit range) presented against our usual rod-suppressing 430 nm background (see Materials and Methods). In its main features it resembles families of current responses from isolated red-sensitive cones of the tiger salamander published by Perry and McNaughton (1991). One difference is that we found little indication of the anomalous increase in time-to-peak with increasing flash intensity described by these authors. This may be due to the low temperature (12°C) in our experiments, whereby response speed may be limited by the kinetics of transduction rather than by cell capacitance (cf. Lamb, Matthews & Murphy, 1989). The dim-flash response in Fig. 1 peaks at 0.2 sec, which is typical of the values found in 20 retinas (mean, 0.21 sec; range, 0.13–0.27 sec) and about twice the time-to-peak of dim-flash responses of dark-adapted salamander cones at 18–22°C (Perry & McNaughton, 1991). Another difference is that our responses to strong flashes did not quite return to baseline for several seconds, indicating a small glial contribution (see below).

Figure 1(B) shows the stimulus–response function derived from the responses in Fig. 1(A). The function is well described by the Michaelis relation

$$U/U_{max} = I/(I + I_0) \quad (1)$$

where I_0 is the half-saturating flash intensity. The crisp saturation behaviour contrasts with *rod* ERG responses, which continue to grow with rising stimulus intensity far beyond the point where the response of the rods themselves saturates. This is probably due to secondary glial (Müller cell) currents induced by extracellular ionic changes even when synaptic transmission is blocked. The good fit of equation (1) to cone stimulus–response data suggests that glial currents do not significantly contribute to response amplitude (see also below).

Although the kinetics of responses clearly suggests cone origin, this can be assessed with confidence only from the spectral sensitivity. Figure 1(C, D) shows superimposed pairs of dim-flash [Fig. 1(C)] and near-saturated [Fig. 1(D)] responses elicited with red (642 nm) and green (519 nm) flashes, matched to be equivalent according to the frog's combined cone sensitivity (Granit, 1942; Reuter, 1969). The green light would then excite red rods (λ_{max} = 502 nm) about 100 times more strongly than the red light. These cone-matched flashes indeed elicited very similar dim-flash responses, indicating that the response to the red stimulus originates practically exclusively in the cones. With strong flashes, the "green" response was larger and returned more slowly to baseline than the "red" response. The difference [third trace in Fig. 1(D)] looks very much like a saturated rod ERG response, suggesting that the background did not fully suppress rods (red and/or green). Note, however, that the contribution of this signal to "red" responses would be more than one order of magnitude smaller than the difference response shown in Fig. 1(D).

The dim-flash response waveform was well fitted by the "independent activation" model used by Baylor, Hodgkin and Lamb (1974) to describe responses of single turtle cones. The response $U(I, t)$ to an "infinitely brief" flash is

$$U(I, t) = an\tau^{-1}Ie^{-t/\tau}(1 - e^{-t/\tau})^{n-1} \quad (2)$$

where a is a sensitivity constant, n is the number of steps in the activation chain, τ is the largest of the time constants (in an arithmetic sequence), I is flash energy (intensity times duration), and t is time after flash. Our flashes were not "infinitely" brief and when fitting the model, $t = 0$ was taken as the midpoint of the flash; further, correction has to be made for a delay always introduced by analogue filtering (see Materials and Methods). As shown in Fig. 1(E), the fit is then good save for (occasionally) a slight elongation of the tail suggestive of some glial intrusion. [In transretinally recorded *rod* mass responses, the later parts tend to be particularly inflated compared with the waveform of single-cell current responses (cf. Donner & Hemilä, 1985; Donner *et al.*, 1988)]. In five retinas where response kinetics were studied with high resolution (see Materials and Methods) the best fits to dim-flash responses indicated a mean number of stages n = 5.6 (range 4–7) in equation (2), not significantly different from the values n = 6 or 7 that Baylor *et al.* (1974) used to fit membrane voltage responses in turtle cones.

The saturated response amplitude grows with rising pH

It was necessary first to establish whether cones (like rods) show a monotonic increase of the saturated response amplitude U_{max} with rising pH_i (in the physiological range). We therefore recorded changes in U_{max} while changing pH_o. Such experiments should in fact also reveal the qualitative dependence of U_{max} on *intracellular* pH (pH_i), because when H^+-transporting mechanisms are intact, pH_i can be assumed at least transiently to change with changing pH_o (see Kalamkarov *et al.*, 1993). It should be noted, though, that our experiments do not provide quantitative information on the dependence of U_{max} on pH_i. The degree of pH_i change will depend on the effective concentration of intracellular buffers and the efficiency of pH-regulating transport mechanisms (both unknown to us).

In Fig. 2 (illustrating one of six similar experiments) a monotonic relation between U_{max} and pH_o is seen to hold over the range 6.9–8.4. Thus cones behaved like rods, but U_{max} changes tended to be less transient than in isolated rods (cf. Koskelainen *et al.*, 1993). In all retinas, lowering pH_o from 7.5 decreased U_{max}, and elevating pH_o from 7.5 increased U_{max}. Particularly the increases upon alkalinization were quite sustained (as in Fig. 2). In two of the retinas, however the increase following pH_o elevation to 7.8 was transient, and further elevation initiated an irreversible decreasing trend of U_{max}. Likewise, strong acidification caused irreversible changes as exemplified in Fig. 2 by the incomplete recovery when pH_o was raised to 7.5 after perfusion at 6.9.

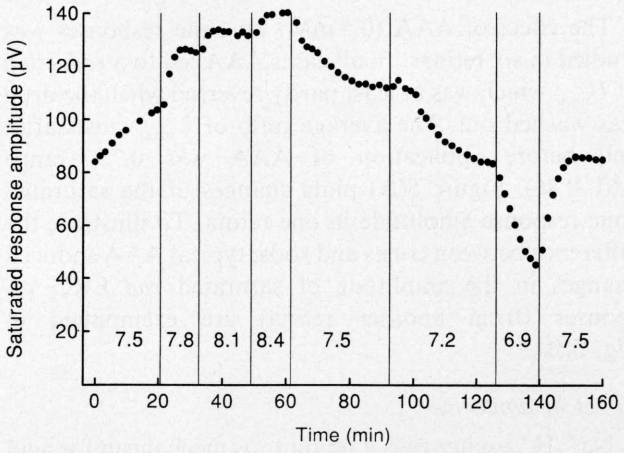

FIGURE 2. The time–course of changes in the saturated response amplitude following changes of pH_o (pH-values indicated between the vertical lines that mark switches). Bicarbonate Ringer.

Bicarbonate transport

Our previous work on rods led to the conclusion that bicarbonate transport is of central importance for maintaining pH_i (Donner *et al.*, 1990; Kalamkarov *et al.*, 1993; Koskelainen *et al.*, 1993). Here we present results of similar experiments on cones, indicating that bicarbonate import, of which at least one component is chloride-coupled, is essential for preventing acidosis.

Increasing $[HCO_3^-]_o$ increases U_{max}. If bicarbonate transport into cones counteracts acidification, then increasing extracellular bicarbonate should increase U_{max}. We varied $[HCO_3^-]_o$ by switching between bicarbonate Ringer (6 mM HCO_3^- + 6 mM HEPES) and nominally bicarbonate-free HEPES Ringer (12 mM HEPES). In all five retinas where this experiment was done, U_{max} increased upon HCO_3^- introduction and decreased upon HCO_3^- withdrawal.

The first part of the experiment in Fig. 3(A) shows the drop and subsequent recovery of U_{max} when bicarbonate was first removed and then reintroduced. The mean ratio of U_{max} values in bicarbonate and HEPES Ringer in the five retinas where this comparison was made was 1.8 (range 1.7–2.2).

DIDS decreases U_{max}. The second type of experiment done to reveal the role of bicarbonate transport was to try to inhibit the transport mechanism(s) with the commonly used anion transport blocker DIDS (0.1 mM). In Fig. 3, the later parts of (A) and (B) illustrate DIDS effects. In bicarbonate Ringer DIDS set off a strong and apparently continuous decrease of U_{max}. The effect was essentially reversible: washing out the drug restored U_{max} to nearly its original value. The results of five similar experiments were entirely consistent, supporting the notion that DIDS acidifies cones by inhibiting a mechanism that transports bicarbonate into the cells. DIDS was effective also in HEPES Ringer [later part of Fig. 3(B)] which suggests that even in nominally bicarbonate-free perfusion, retinal metabolism keeps endogenous HCO_3^- in the tissue high enough to be important for pH_i regulation.

Decreasing $[Cl^-]_o$ increases U_{max}. The third type of experiment related to bicarbonate transport was done specifically to reveal whether a chloride-coupled exchanger (HCO_3^-/Cl^-) is involved. If so, increasing the outward driving force for chloride by lowering $[Cl^-]_o$ should accelerate the inward transport of bicarbonate, thus elevate pH_i and increase U_{max}. We reduced $[Cl^-]_o$ by substituting the 95 mM NaCl in the Ringer by sodium-gluconate (taking care to keep free Ca^{2+} constant, see Materials and Methods). This substitution indeed caused a significant growth of U_{max} in all three retinas where it was carried out. However, as shown by the example in Fig. 4, the increase was transient, indicative of negative feedback acting to restore pH_i to its original value. One possibility is that the high pH_i inactivates other (possibly all) acid-extruding mechanisms. This explanation would be consistent with the large negative transients seen upon return to normal chloride.

Carbonic anhydrase

In studies on rods in the same isolated-retina preparation as used here, we found that inhibiting retinal carbonic anhydrase (CA) by acetazolamide (AAA)

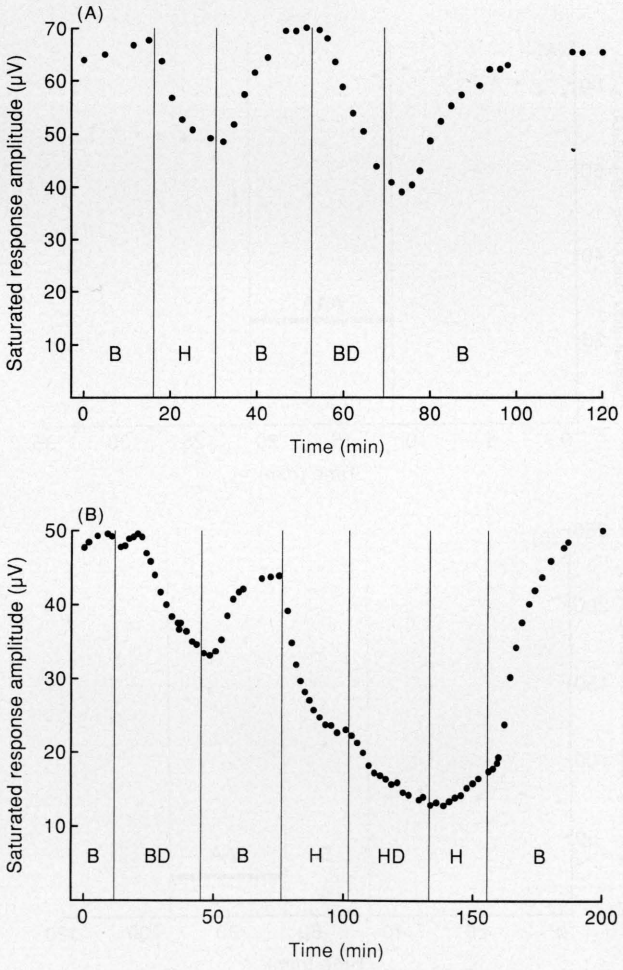

FIGURE 3. Effects of changes in HCO_3^- concentration and addition of 0.1 mM-DIDS in two retinas (A, B). In both panels, the perfusion sequence is indicated by letters between the vertical lines that mark perfusate switches: B, bicarbonate Ringer; H, HEPES Ringer; BD, bicarbonate Ringer + DIDS; HD, HEPES Ringer + DIDS.

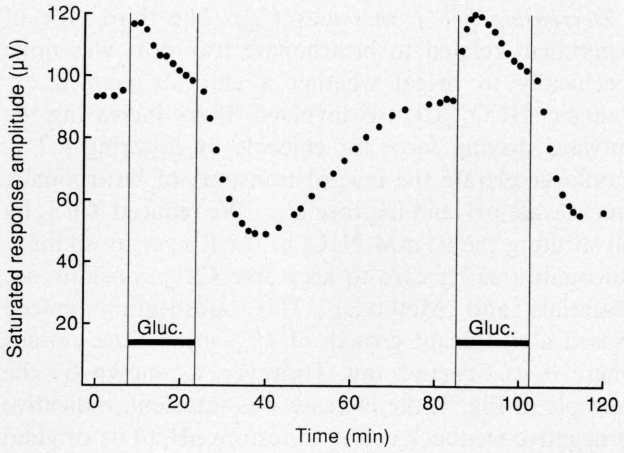

FIGURE 4. The effect of substituting 95 mM NaCl by sodium gluconate. The periods in gluconate are indicated by horizontal bars. Bicarbonate Ringer.

increased photoresponse amplitudes (Donner *et al.*, 1990). This is not a direct effect on the rods (which lack CA), but could be due to a change in the flux of H^+ and HCO_3^- through the retina (see Discussion). Obviously, the situation might be different for cones, which possess CA (Musser & Rosen, 1973; Linser & Moscona, 1984).

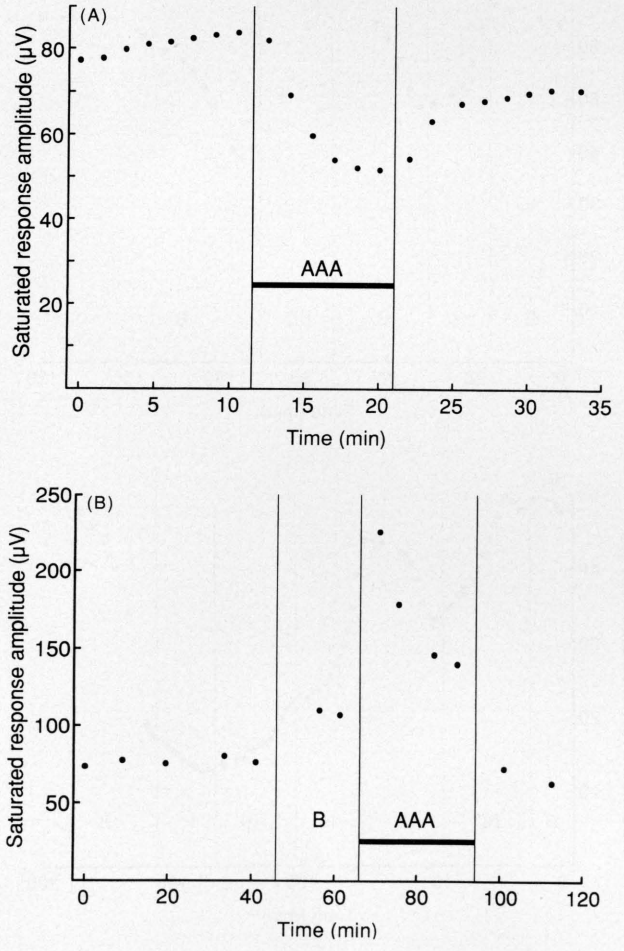

FIGURE 5. Effect of 0.5 mM-acetazolamide on the saturated response amplitude of cones (A) and rods (B). Horizontal bars (AAA) show the periods of drug application. Bicarbonate Ringer in (A). In (B), the effect of introducing 6 mM bicarbonate into initially nominally bicarbonate-free Ringer is also seen (at *c.* 45 min).

The effect of AAA (0.5 mM) on cone responses was studied in six retinas. In all cases AAA led to a *reduction* of U_{max}, which was at least partly reversed when the drug was washed out. The average ratio of U_{max} values after and before application of AAA was 0.75 (range 0.61–0.86). Figure 5(A) plots changes of the saturated cone response amplitude in one retina. To illustrate the difference between cones and rods, typical AAA-induced changes in the amplitude of saturated *rod* ERG responses (from another retina) are exemplified in Fig. 5(B).

Effect of amiloride

Na^+/H^+ exchange is a ubiquitous mechanism for acid extrusion in animal cells. It is commonly studied by means of the inhibitor amiloride, but this drug is problematic in physiological experiments on photoreceptors because of the profound effect of simultaneous inhibition of $Na^+/Ca^{2+},K^+$ exchange (Benos, 1988; Koskelainen *et al.*, 1993). We still thought it worthwhile to try amiloride, particularly so as the alternative strategy of removing Na^+ would have even more obnoxious effects. Amiloride (2 mM) was applied to five retinas. In all cases, it triggered a decay of U_{max}, which was partly reversed when the drug was washed off (Fig. 6). In three of the experiments, there was a substantial (several minutes) delay before the drug took visible effect. This is noteworthy, because inhibition of $Na^+/Ca^{2+},K^+$ exchange would be expected to be evident "immediately", given our time resolution (Hodgkin, McNaughton & Nunn, 1985). Thus, although not compelling, the results support a role of Na^+/H^+ exchange in cone pH_i regulation.

DISCUSSION

Acid removal by bicarbonate transport in cones and rods

Intracellular pH (pH_i) depends on a balance of H^+ production and removal. Photoreceptor cells have a high rate of metabolic acid production in the inner segment (Winkler, 1981, 1986) and particularly under

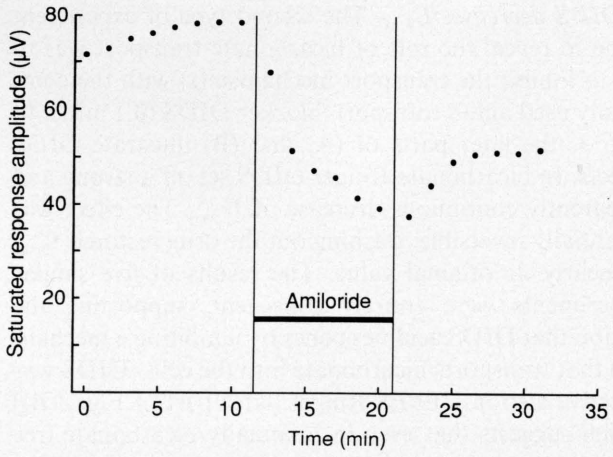

FIGURE 6. Effect of 2 mM-amiloride on the saturated response amplitude. The horizontal bar shows the period of amiloride application. Bicarbonate Ringer.

illumination large amounts of H^+ are released in the outer segment by cGMP synthesis and hydrolysis (Liebman *et al.*, 1984; Pugh & Lamb, 1990). pH_i cannot be allowed to drop too much, as this inactivates phototransduction at least in rods (Sillman, Owen & Fernandez, 1972; Gedney & Ostroy, 1978; Liebman *et al.*, 1984; Meyertholen, Wilson & Ostroy, 1986; Donner *et al.*, 1990). Around neutral pH_i, however, acid removal by passive diffusion (of neutral CO_2 or lactic acid, which cross cell membranes) would be small unless supported by ion exchangers, coporters or pumps. There is thus an evident need for such transport mechanisms in photoreceptor cells. On the other hand, of course, (controlled) changes in pH_i could fulfil a functional role in modulating phototransduction.

Acid can be removed not only by direct H^+-extrusion (e.g. Na^+/H^+ exchange, cf. Fig. 6), but also by HCO_3^--import. This ion then captures H^+ to liberate CO_2, which diffuses out. The present results strongly support the idea that bicarbonate transport is important for the maintenance of physiological pH_i in cones, and that at least part of this transport involves HCO_3^-/Cl^- exchange. The sodium-dependent bicarbonate-chloride ($Na^+/H^+/HCO_3^-/Cl^-$) exchanger could in principle explain most of the results. Being coupled to the strongly inward sodium gradient, it will (when active) always drive bicarbonate ions into the cell and elevate pH_i. Inhibition would always depress pH_i and U_{max}, as was observed upon application of DIDS. This would not in general be true of the sodium-*in*dependent HCO_3^-/Cl^- exchanger, which will act to stabilize pH_i at thermodynamic equilibrium (cf. Kalamkarov *et al.*, 1993). Still, we have no evidence against other types of bicarbonate transporters, e.g. sodium-bicarbonate coporters.

The different roles of carbonic anhydrase for cones and rods

Application of the CA-inhibitor AAA always reduced the amplitude of cone responses. This contrasts with the situation for rods in the same preparation, where AAA always raised the levels of cyclic GMP and boosted photoresponses (Donner *et al.*, 1990). As rods are thought to lack CA, the effect on rods is best interpreted as an indirect one due to CA inhibition in the inner retina [decreased transport of H^+ to the photoreceptor layer, rising level of extracellular bicarbonate, both leading to relative *alkalinization* (see Donner *et al.*, 1990)]. Obviously, the very same changes in the extracellular environment will be experienced by the cones, so if other things were equal, an *in*crease in photoresponse amplitude would be expected, instead of the decrease observed.

It is natural to assume that the difference is related to the presence of CA in COS as opposed to ROS. It is possible that in COS bicarbonate import is the dominating mechanism for acid removal, being efficient enough thanks to the presence of CA, which ensures a fast equilibrium between CO_2 and H^+-HCO_3^-. Inhibition of CA would then decrease the efficiency of extrusion, acidify the COS and cause a drop in U_{max}. In rods, on the other hand, the absence of CA implies that bicarbonate-based H^+ removal can deal but slowly with changes, and indeed, ROS appear to extrude acid chiefly by Na^+/H^+ exchangers (Koskelainen *et al.*, 1993).

It is possible to envision a positive advantage for the COS from removing acid by CO_2 diffusion rather than H^+ export. The "external" space is large in relation to the total volume of COS and is not known to contain CA [whether in ROS or extracellularly (see however Kaila, Paalasmaa, Taira & Voipio, 1992)]. Thus CO_2 may diffuse far (e.g. into the pigment epithelium) before being hydrated to impose an acid load. By contrast, direct extrusion of H^+ ions would inevitably to some extent lead to H^+-accumulation on the outer surface of the membrane, increasing the gradient against which further extrusion must work. The dense folding of the plasma membrane and high surface/volume ratio of COS would make this a more serious problem in cones than in rods (i.e. the COS membrane folds would become acidic).

The inner segment

The above considerations refer to pH_i control in the *outer* segment. A tacit assumption is that H^+ diffusion between the inner and outer segment is small compared with H^+ turnover in either segment alone, so the two can be treated more or less separately (see Koskelainen *et al.*, 1993). In the inner segment, there is continuous acid production by energy metabolism (Winkler, 1981, 1986). We have found that acidification of the inner segment in *rods* abolishes photosensitivity, probably via depression of metabolism (Koskelainen *et al.*, 1993), and that pH_i maintenance depends strongly on bicarbonate. The task and needs of cone inner segments would not *a priori* be expected to differ significantly from those of rod inner segments, nor do our present results indicate such differences.

CONCLUSION

In most respects, our results on the physiological role of pH-regulating mechanisms in cones resemble those previously obtained from rods. The main difference is that bicarbonate transport seems to play a somewhat different and possibly even more important role. When related to cone/rod differences in morphology and physiology and particularly to the presence of carbonic anhydrase in cone outer segments, it suggests that cones remove their acid largely by CO_2 diffusion.

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