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ROD PHOTOTRANSDUCTION MODULATED BY BICARBONATE IN THE FROG RETINA: ROLES OF CARBONIC ANHYDRASE AND BICARBONATE EXCHANGE

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SUMMARY

1. Effects on rod phototransduction following manipulation of retinal CO_2–HCO_3^- and H^+ fluxes were studied in dark-adapted retinas of the frog and the tiger salamander.

2. Rod photoresponses to brief flashes of light were recorded from the isolated sensory retina as electroretinogram mass receptor potentials and from isolated rods by the suction-pipette technique. The experimental treatments were: (1) varying $[CO_2]+[HCO_3^-]$ in the perfusion fluid; (2) applying acetazolamide (AAA), which inhibits the enzyme carbonic anhydrase (CA); and (3) applying 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) which blocks exchange mechanisms transporting HCO_3^- across cell membranes.

3. The concentration of the internal transmitter of the rods, cyclic GMP, was biochemically determined from the rod outer segment layer of retinas that had been incubated in the same solutions as were used for perfusion in the electrophysiological experiments.

4. The introduction of 6 mM-sodium bicarbonate to replace half the buffer of a nominally CO_2–HCO_3^--free (12 mM-phosphate or HEPES, $[Na^+]$ constant) Ringer solution doubled the cyclic GMP concentration in the rod outer segment layer and increased the saturating response amplitude and the relative sensitivity of rods in the intact retina.

5. The introduction of 0·5 mM-AAA into bicarbonate-containing Ringer solution accelerated the growth of saturated responses and sensitivity. Incubation of the retina in AAA–bicarbonate Ringer solution elevated the concentration of cyclic GMP ninefold compared with the phosphate control.

6. No effects of switching to bicarbonate–AAA Ringer solution were observed in the photocurrent of isolated rods drawn into suction pipettes with only the outer segment protruding into the perfusion fluid. The target of AAA is probably the CA-containing Müller cell.

7. The introduction of DIDS into the perfusate (at normal pH 7·5) set off a

continuous decay of photoresponses which finally abolished light sensitivity completely. The decay proceeded regardless of whether bicarbonate and AAA were present or not.

8. Rods that had lost their photosensitivity in DIDS recovered almost fully when the pH of the DIDS perfusate was raised to 8·5. They also recovered when DIDS was washed out with bicarbonate Ringer solution at constant pH (7·5).

9. It is proposed that all our treatments ultimately modulate the intracellular pH of the rods which is determined by the relative rates of H^+ leakage and HCO_3^- transport into the cells. The rates depend on the concentrations of these ions around the rods; AAA probably acts by slowing down the acid transport from the inner retina by Müller cells. Intracellular pH is an important determinant of both the amplitude and the kinetics of rod photoresponses.

INTRODUCTION

Phototransduction in retinal rods is sensitive to pH. Acid perfusion both decreases the light-sensitive current (Sillman, Owen & Fernandez, 1972; Gedney & Ostroy, 1978) and slows down response kinetics (Liebman, Mueller & Pugh, 1984). When retinas are incubated in media of low pH buffering capacity, which causes acidification of the retina (Oakley & Wen, 1989), the cyclic GMP content and light sensitivity of the rods are depressed (Meyertholen, Wilson & Ostroy, 1980, 1986).

The retinal tissue in which the rods are embedded has a high metabolic rate (Warburg, 1926; Futterman & Kinoshita, 1959) and sparse vascularization. The stable functioning of phototransduction would thus seem to require efficient mechanisms to deal with metabolic CO_2 and H^+. These two are inextricably linked because after hydration carbon dioxide will liberate hydrogen ions and because CO_2–HCO_3^- constitutes a major physiological pH buffer. In the experiments to be described, rod phototransduction was studied in conjunction with treatments selected to affect hypothesized mechanisms for the retinal transport of CO_2–HCO_3^-–H^+ and the regulation of intracellular pH.

Several works have suggested the importance of bicarbonate for maintaining the responsiveness of the retina (Meyertholen *et al.* 1980, 1986; Winkler, 1986; Oakley & Wen, 1989). However, in these works the introduction of bicarbonate has generally been accompanied by significant increases in buffering capacity, so the role of the HCO_3^- ion itself has remained unclear. We find that the introduction of CO_2–HCO_3^- boosts photoresponses and increases the concentration of cyclic GMP in the rods even when the H^+-buffering capacity of the perfusate has not changed. This implicates a specific HCO_3^--dependent mechanism in the rods. Many neurons and glial cells are known to regulate intracellular pH by bicarbonate-dependent exchangers, transporting HCO_3^- into cells (see for example Russell & Boron, 1976; Thomas, 1977, 1984; Chesler, 1986; Schlue & Deitmer, 1988). We therefore investigated whether such an exchanger is active in rods by administering the disulphonic stilbene DIDS, commonly used to inhibit anionic exchange mechanisms. The results indicate that a DIDS-inhibitable mechanism is indeed essential for maintaining normal phototransduction.

The acid load experienced by rods depends largely on metabolism and transport

in other retinal cells. Two recent studies with H^+-selective electrodes have indicated that the efficiency of H^+ buffering and removal in the retina depends on the activity of the enzyme carbonic anhydrase (CA) (Borgula, Karwoski & Steinberg, 1989; Oakley & Wen, 1989), which catalyses the reaction of CO_2 with water and thus ensures a fast equilibrium in the CO_2–HCO_3^-–H^+ system. Carbonic anhydrase might then contribute to the regulation of $[H^+]$ and $[HCO_3^-]$ around rods, although the rods themselves lack the enzyme (Musser & Rosen, 1973; Linser & Moscona, 1984). In agreement with this, we found substantial effects on phototransduction in experiments where retinal CA was inhibited with acetazolamide (AAA).

Our general conclusion is that the maintenance of rod intracellular pH (pH_i) at a level optimal for phototransduction is critically dependent on the availability of HCO_3^- around the rods. The relations between rods and Müller cells which we propose to account for the results are schematically summarized in Fig. 8 in the Discussion.

METHODS

Electroretinogram (ERG) recording across the perfused sensory retina

Since it was desirable to keep the rods themselves, their packing in the retinal tissue, and their interactions with the Müller cells essentially intact, the preparation mainly used in this work was the perfused isolated retina. The rod photocurrent was recorded as an aspartate-isolated ERG mass receptor potential across the isolated retina of the frog, *Rana temporaria* (caught in the wild in southern Finland) or the tiger salamander, *Ambystoma tigrinum* (Carl Lowrance Waterdog Farm, Tulsa, OK 74136, USA). The techniques are described in greater detail by Donner & Hemilä (1985) and Donner, Hemilä & Koskelainen (1988). The animals were killed and double-pithed, the eyes were excised, and the retina of one eye was isolated in cooled Ringer solution. The retina was mounted receptor-side upwards in a specimen holder. In the present context, it is important to note that the geometry prevented substances from escaping at the vitreal side. The transretinal voltage was DC-recorded with Ag–AgCl electrodes and stored in digital form (digitization at 100 Hz with two-point smoothing in most cases). In order to facilitate the comparison of responses, the long-term baseline drift (if any) has been subtracted in the figures shown in this paper.

The receptor side of the retina was perfused with Ringer solution containing 2 mM-aspartate, sufficient to block synaptic transmission from receptors to higher-order neurons. The temperature in these experiments was 14 °C. The stimuli in both these and the experiments on isolated rods were 67 or 100 ms flashes of 493 nm light. Flash intensities are given as photoisomerizations per rod, denoted Rh*.

Current recording from isolated rods

In a smaller number of experiments, the current of isolated rods of the tiger salamander (*Ambystoma tigrinum*) was recorded by the suction-pipette method of Baylor, Lamb & Yau (1979) with details as described by Donner, Hemilä & Koskelainen (1989). A suitable rod was selected under infra-red video inspection and the inner segment was drawn into the glass micropipette so that the rod current had to pass through a current-to-voltage converter. The voltage signal was amplified, digitized and stored on diskette. The outer segment projecting out of the pipette was perfused with Ringer solutions of desired composition. These recordings were done at 21 °C.

Perfusion

The perfusion system was gravity controlled and, when solutions were switched, one replaced another in less than one minute. The Ringer solutions each contained (in mM): 95 NaCl, 3 KCl, 0·5 $MgCl_2$, 0·9 $CaCl_2$, 10 glucose and 2 sodium aspartate, and were buffered to pH 7·5 unless stated otherwise. The pH buffer was either (1) 12 mM-sodium phosphate (phosphate Ringer solution), (2) 6 mM-sodium phosphate plus 6 mM-sodium bicarbonate (bicarbonate Ringer solution), (3) 12 mM-HEPES (HEPES Ringer solution), or (4) 6 mM-HEPES plus 6 mM-sodium bicarbonate (HEPES–bicarbonate Ringer solution). When used together with other buffers, bicarbonate buffer at this

low concentration and high pH requires no CO_2 bubbling. CO_2 escapes very slowly, as was checked by continuous pH measurements in a Ringer solution buffered with only 6 mM-bicarbonate with no phosphate or HEPES and stored in an open beaker. Thus we know that the bicarbonate Ringer solutions we used remained essentially stable for the duration of the experiments. Care was taken to keep the Na^+ concentration equal in all solutions.

Acetazolamide (AAA) is a powerful inhibitor of CA and considered to be quite specific when applied in concentration below millimolar (Maren, 1967, 1977). On the other hand, at least 95–99 % inhibition of the enzyme is generally required for significant physiological effects to appear (Sapirstein, 1983). Therefore, we chose to use the comparatively high concentration 0·5 mM-AAA (Sigma) added to the Ringer solution in most experiments in the present study.

4,4′-Diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) is commonly used to inhibit anionic exchange mechanisms, including those that are HCO_3^- dependent (see Thomas, 1984; Passow, 1986). Since non-specific effects of this and the related stilbene 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid (SITS) have been reported at higher concentrations (Biagi, 1985; Wieth & Brahm, 1985; Curci, Debellis & Frömter, 1987) we used, as a rule, the concentration 0·1 mM-DIDS (Sigma) added to the Ringer solution. In fish red blood cells this concentration blocks anion transport completely, but largely reversibly, even after 1 h perfusion (Pasternack, 1988; and M. Nikinmaa, personal communication). In a few experiments where we wanted to see if DIDS would take effect faster with higher concentrations we used 0·5 mM (with no obvious difference).

Determination of the cyclic GMP concentration in rod outer segments

Isolated frog retinas were incubated in darkness in Ringer solutions of the same compositions as used in the electrophysiological experiments (see above). The retinas were then rapidly frozen in dim red light on a microtome table and sectioned in 15 μm slices (see Zak, Lelekova & Ostrovskii, 1974). The purity of the preparation was checked by electrophoresis in polyacrylamide gel (PAGE) (Görg, Postel, Weser, Shiwara & Boesken, 1985). Rhodopsin, G-protein and arrestin were the only major proteins, indicating that the preparation consisted almost exclusively of rod outer segments (see Fig. 3 below). After sectioning, 0·5 ml of 30 % trichloroacetic acid was added in order to stop enzymatic reactions, and the sample was centrifuged at 10000 *g* for 10 min. It was then filtered through a paper filter and the filtrate was assayed for cyclic GMP content using the radioimmunoassay technique of Steiner, Darker & Kipnis (1972). The protein content of the slices was determined spectrophotometrically at 280 nm after dissolving in 0·1 % cetyltrimethylammonium bromide.

Analysis

When considering effects on photoresponses, it is generally useful to distinguish changes in the amplitude of the saturated response (*maximum response* amplitude, here denoted U_{max}), which indicates the size of the rod's light-sensitive current, from changes in the *relative sensitivity* (here denoted S), which expresses how large a fraction of that current is turned off by one photoisomerization. Obviously the two reflect rather different physiological mechanisms. The amplitude (here denoted U) of a response to a weak flash (producing I photoisomerizations in the rod) gives the *absolute* sensitivity $S_a = U/I$ of the rod, i.e. response amplitude per photoisomerization. The relative sensitivity S is the ratio of S_a and U_{max} ($S = S_a/U_{max}$). In practice, we separated U_{max} and S by fitting a type of generalized Michaelis stimulus–response curve (z-function, see Bäckström & Hemilä, 1979) to response amplitudes covering the intensity range from dim to near-saturating. The position of the curve on the log intensity axis gives log S, and its position on the log response amplitude axis gives log U_{max}.

RESULTS

Bicarbonate boosts photoresponses

To clarify the role of the bicarbonate ion as such, we first studied changes in rod photoresponses when retinal $[CO_2]+[HCO_3^-]$ was directly increased. This was done by replacing half the initial 12 mM-phosphate or HEPES buffer with sodium bicarbonate (6 mM). It is worth noting that (1) this partial replacement of phosphate

by bicarbonate buffer where CO_2 is not kept constant does not increase the H^+-buffering capacity of the perfusate; (2) the retina contains a non-negligible concentration of CO_2–HCO_3^- even in nominally bicarbonate-free perfusion, but the concentration will necessarily rise when bicarbonate is added as diffusion out of the retina is retarded; and (3) although CO_2 enters the rod unimpeded and is hydrated, the buffering capacity of a rod is so high (Dearry, 1981, cited by Liebman *et al.* 1984) that no significant intracellular acidification is expected from CO_2 entry at the low concentration involved here.

The retina was first allowed to stabilize in phosphate (or HEPES) perfusion for 1–1·5 h after dissection. When the perfusion was thereafter switched to bicarbonate Ringer solution the responses immediately started to grow. In Fig. 1, panels *A*, *C* and *D* each show a set of three responses to a constant stimulus intensity which was low in *A*, intermediate in *C* and high in *D*. The smallest of the responses in each set was recorded first in nominally bicarbonate-free phosphate perfusion, while the middle responses show the situation *ca* 15 min after the switch to bicarbonate perfusion. (The largest response in each panel was recorded after the subsequent addition of AAA and will be considered further below).

After 15 min in bicarbonate, near-saturated responses (panel *D*) had grown by about 40%. However, dim-flash responses (panel *A*) had grown even more (relatively), indicating an increase in relative sensitivity. In eight experiments where half of the 12 mM-phosphate buffer was thus replaced by 6 mM-bicarbonate, the amplitude of saturated responses grew on average by a factor of 1·3 (range 1·0–1·7) and relative sensitivity by a factor of 1·6 (range 0·9–3·2).

The bicarbonate effects are enhanced by the inhibition of carbonic anhydrase

Effects of AAA

The addition of 0·5 mM-AAA to the perfusate, when bicarbonate was already present, brought an accelerated growth of photoresponses to both dim and bright flashes. In Fig. 1, the largest response in each panel was recorded after the addition of AAA. Compared with the situation in AAA-free bicarbonate Ringer solution, 0·5 mM-AAA boosted U_{max} on average by a factor of 1·4 (range 1·1–2·0) and S by a factor of 1·6 (range 0·8–3·6). It is important to note that, besides showing a rather large variation, the growth factors depended in a systematic manner on the interval between the introduction of bicarbonate and the addition of AAA (15–70 min in these experiments, see below).

The growth of responses is not due to glial currents

The main disadvantage of mass responses (as compared with single-rod recordings) is that their later phases are contaminated by currents which do not originate in the photoreceptors (Sillman, Ito & Tomita, 1969). It might then be argued that the large responses obtained in bicarbonate and AAA in Fig. 1 do not really reflect a growth of the rod photocurrent, but rather an enhancement of secondary currents (mainly of glial origin). To rule out this possibility, the early rises of the near-saturated responses of Fig. 1*D* are shown in *B* on expanded scales. As synaptic transmission is blocked, non-receptor currents, caused by extracellular ionic changes attending the

photoreceptor response, can contribute to the ERG potential only after a delay. However, the percentage growth of photoresponses induced by bicarbonate and AAA is seen to be even larger at the very first deflection from the baseline than at the peak of the response (Fig. 1*B vs. D*).

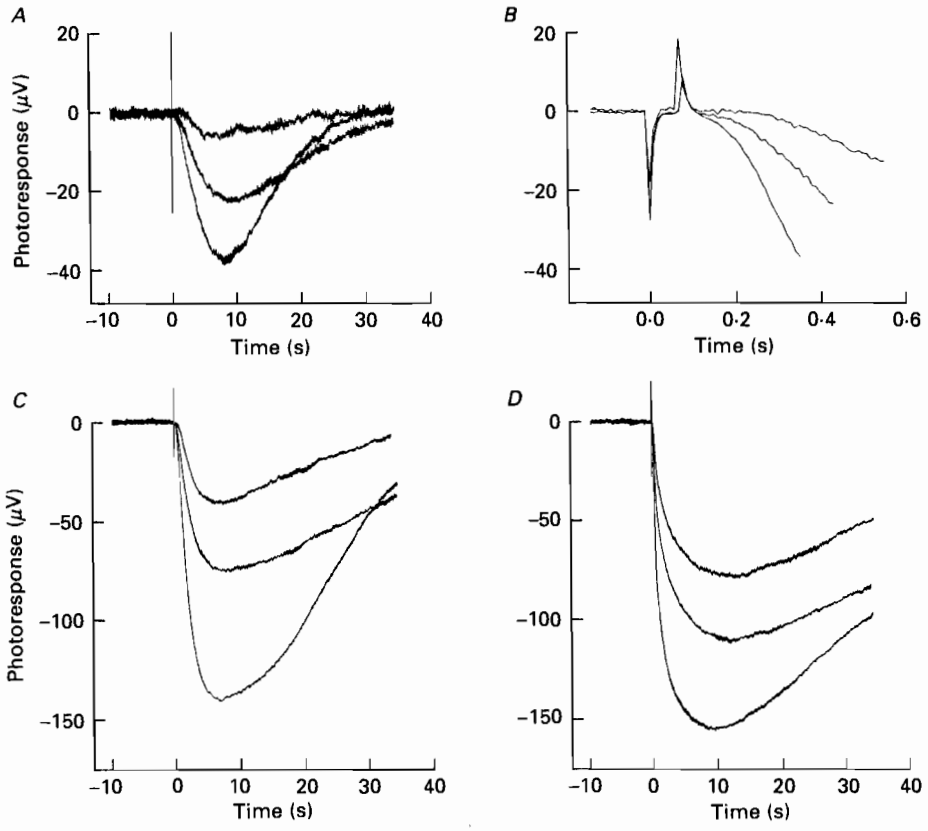

Fig. 1. The effects of CO_2–HCO_3^- and acetazolamide (AAA) on mass receptor responses from the isolated frog retina. Each panel shows three responses to the same flash intensity, the first (smallest) one recorded in phosphate, the second (middle) one recorded in bicarbonate, and the third (largest) one recorded after the addition of 0·5 mM-AAA to the bicarbonate Ringer solution. Note that the amplitude scales in *C* and *D* are different from those in *A* and *B*. The flash intensities, expressed as numbers of photoisomerizations per rod, were: *A*, $I = 1{\cdot}1$ Rh*; *C*, $I = 23$ Rh*; *D*, $I = 460$ Rh*. *B*, the initial rises of the saturated responses in panel *D* shown on expanded scales. The downward and upward spikes preceding the responses indicate the onset and the offset of the flash. See text for details.

Response kinetics are accelerated in bicarbonate–AAA

Rod responses to dim flashes, as well as the earliest rise of responses to brighter flashes, depend linearly on flash intensity (Baylor *et al.* 1979; Donner, 1989). If response kinetics were not changed by bicarbonate and AAA the response growths in Fig. 1*A* and *B* would both equally express the increase in the absolute sensitivity of the rod. In fact, the peak amplitude of the dim-flash response (panel *A*) is 6 times larger in bicarbonate–AAA than in phosphate, while the corresponding amplitude

ratio during the early rise of the bright-flash responses (panel *B*) is about 10. This difference indicates that responses not only grow in bicarbonate and AAA, they also rise more steeply.

Dependence of AAA action on the bicarbonate regime

The similar effects of bicarbonate and AAA (Fig. 1) would be most parsimoniously explained by assuming that the action of both share a common final pathway (such as the modulation of pH_i). This idea is supported by the fact that AAA effects were highly dependent on the bicarbonate history. If AAA was introduced into nominally bicarbonate-free Ringer solution, the effects were weak or could even be completely missing (four experiments in phosphate and two in HEPES). When AAA was introduced into bicarbonate Ringer solution, there was always some increase of at least the saturating response amplitude, but the magnitude of the effect was generally smaller the longer the time that elapsed between the introduction of bicarbonate and that of AAA. If the retina had spent more than 30 min in bicarbonate, AAA effects were weak. The general impression was that increasing $[HCO_3^-]$ and applying AAA both acted through a common mechanism which reached a 'saturation level' within *ca* 20 min in bicarbonate Ringer solution.

Figure 2 traces the time course of changes in U_{max} and S through the experiment from which the responses in Fig. 1 have been taken. The switch from phosphate (P) to bicarbonate (B) Ringer solution set off a steady increase in the amplitude of saturated responses and elevated relative sensitivity to a new level. The 'bicarbonate' responses shown in Fig. 1 were taken near the end of this stage. After 17 min, AAA was added, producing fairly quick rises in U_{max} and S, both of which, however, were essentially transient. The 'AAA' (largest) responses in Fig. 1 were taken close to the peak of the effect.

The return to phosphate Ringer solution set off a continuous decay of U_{max}, while relative sensitivity fell back to its initial (pre-bicarbonate) value. The subsequent reintroduction of bicarbonate and AAA was able to reverse the decay. The amplitude of dim-flash responses grew by about tenfold (the increase of $\log U_{max} + \log S$ was about 1).

Even in experiments where the initial effects of bicarbonate and/or AAA were modest or absent, a steep decay of U_{max} was always triggered by the return to bicarbonate-free Ringer solution. Likewise, the reintroduction of bicarbonate–AAA invariably brought a significant recovery. In most experiments, the recovery of U_{max} was more complete than is evident from Fig. 2. This emphasizes the essential robustness of the effects, overriding variations in the initial state of individual preparations.

Bicarbonate and AAA increase the concentration of cyclic GMP in the rod outer segments

To establish further the character of bicarbonate and AAA action, we investigated whether they were able to modulate the concentration of the internal transmitter of the rods, cyclic GMP. Values of [cyclic GMP] in the layer of rod outer segments were compared between retinas that had been subjected to six different incubation protocols (see Table 1), each repeated on three retinas. The incubation media were

identical to the perfusates of the electrophysiological experiments, but in this case the retina was floating freely so that both the vitreal and the receptor sides were bathed by the solution. All incubations were done in darkness, and the cyclic GMP content was determined as described in the Methods section. Figure 3 shows the

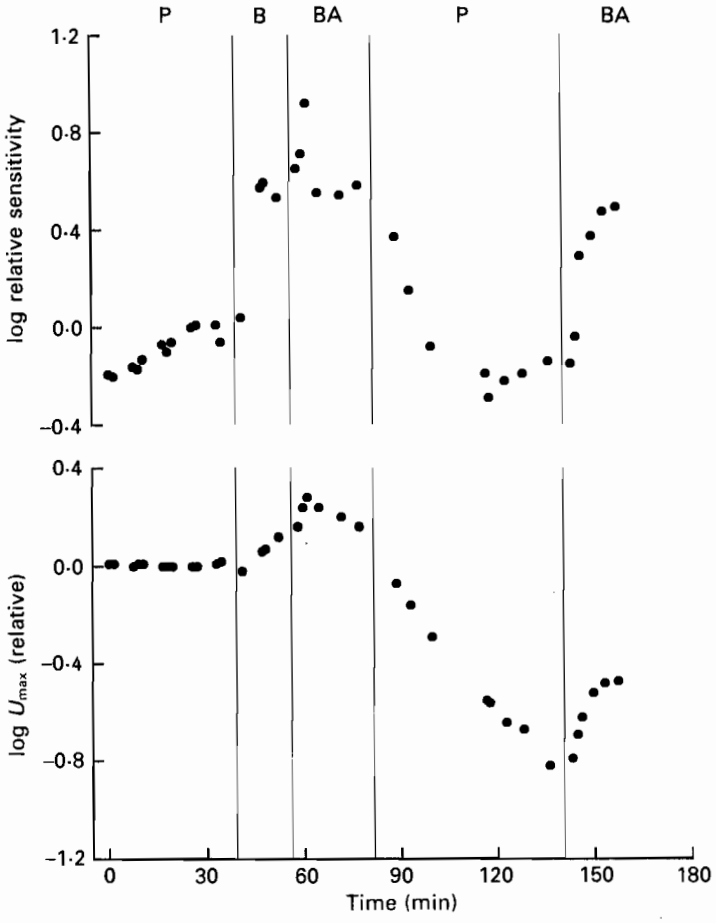

Fig. 2. The time course of changes in the saturated response amplitude U_{max} and the relative sensitivity S (log scale) due to bicarbonate and acetazolamide (AAA). Dim and bright flashes alternated and each dot plots a value of S or U_{max} obtained by fitting a log U–log I template curve (see Methods) to the amplitude of one response taken at the time indicated by the abscissa. In this fitting, the 'missing' parameter (e.g. U_{max} when fitting the curve to the amplitude of a small response) was obtained by interpolation between the immediately preceding and following responses. Perfusion sequence: phosphate (P), bicarbonate (B), bicarbonate–AAA (BA), phosphate (P) and bicarbonate–AAA (BA). The vertical lines give the times of the perfusion changes.

protein composition of the preparations from which [cyclic GMP] was determined. The only major proteins are rhodopsin, G-protein and arrestin, indicating that there is no significant contamination from retinal structures other than rod outer segments.

Table 1 summarizes the results of these experiments. The effects on cyclic GMP

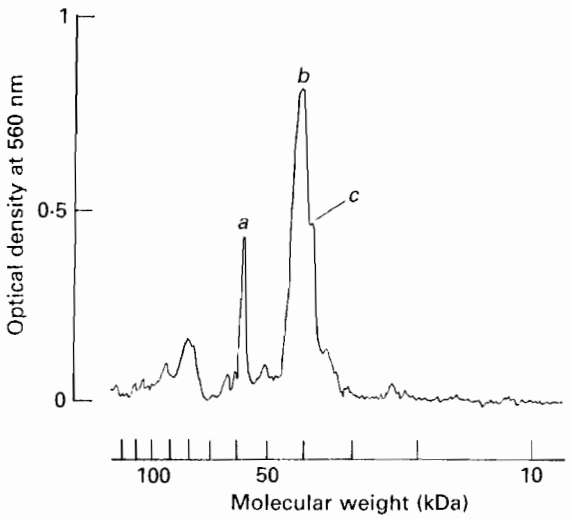

Fig. 3. Sodium dodecylsulphate–10% polyacrylamide gel electrophoresis of rod outer segment slice proteins. Proteins were stained with 'Serva R-250' (Serva, FRG). The most prominent bands determined after a protein marker ('protein test mixture 4', Serva, FRG) were arrestin (*a*), rhodopsin (*b*) and G-protein (*c*).

TABLE 1. Cyclic GMP concentrations in slices of rod outer segment layer after incubation of intact retinas

Number	Incubation treatment	Cyclic GMP concentration (pmol/mg protein)
1	30 min in phosphate Ringer solution	45±4
2	15 min in phosphate Ringer solution, 15 min in bicarbonate Ringer solution	93±6
3	30 min in bicarbonate Ringer solution	94±9
4	30 min in phosphate Ringer solution containing 0·5 mM-AAA	40±3
5	30 min in bicarbonate Ringer solution containing 0·5 mM-AAA	394±9
6	30 min in phosphate Ringer solution containing 5 mM-IBMX	698±13

The values given are means±standard deviations of experiments performed in triplicate. The effect of applying the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) is included to give an idea of the relative magnitudes of the other effects. AAA, acetazolamide.

levels turn out to be qualitatively well correlated with the electrophysiologically observed effects on the size of photoresponses. In phosphate Ringer solution, the addition of AAA caused no increase in [cyclic GMP]. A switch from phosphate to bicarbonate Ringer solution doubled the level of cyclic GMP within 15 min, while 15 min more in bicarbonate produced no additional increase. Introducing AAA into the bicarbonate Ringer solution elevated [cyclic GMP] further by a factor of 4 (making it almost 9 times higher than in the phosphate control).

These experiments provide direct evidence that bicarbonate and AAA applied to intact retinas affect the biochemical machinery in the rod outer segments. The good qualitative correlation between the amplitudes of the electrophysiologically recorded

receptor responses and total [cyclic GMP] is noteworthy. On the other hand, there are important quantitative differences, particularly the very high [cyclic GMP] value in AAA, which should be compared with the moderate and essentially transient growth of photoresponses in AAA (cf. Fig. 2). The reasons for the failure of a quantitative correlation will be considered in the Discussion.

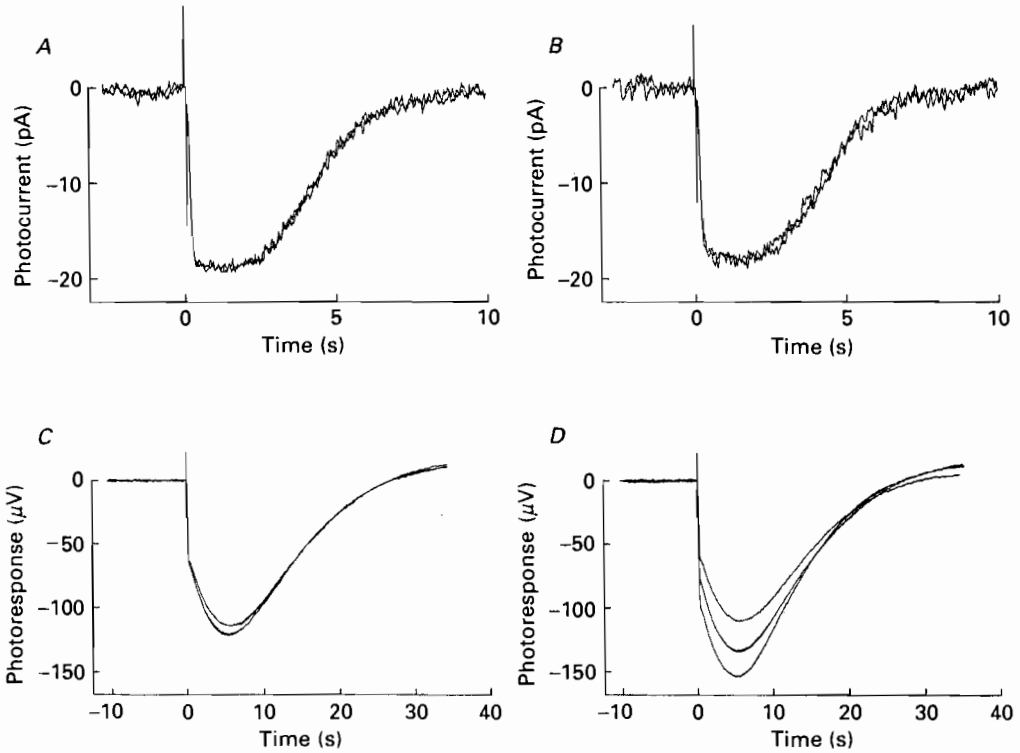

Fig. 4. Absence of effect of bicarbonate–AAA on the photocurrent of an isolated salamander rod (*A* and *B*, 21 °C), contrasted with the effects on the mass receptor potential of the intact salamander retina (*C* and *D*, 14 °C). Eleven-point smoothing applied in *A* and *B*. *A*, two saturated photoresponses in HEPES Ringer solution taken just before the perfusion change. *B*, two responses to the same flash intensity taken 1 min 45 s and 5 min 30 s after the switch to HEPES–bicarbonate–AAA Ringer solution. *C*, two saturated responses in HEPES Ringer solution. The larger response was taken 8 min and the smaller response 1 min 30 s before the change of perfusion, indicating that the response amplitude was decreasing slowly. *D*, three responses to the same flash intensity as in (*C*), taken 1 min (smallest), 7 min (middle) and 15 min (largest) after the introduction of bicarbonate–AAA.

Bicarbonate and AAA do not affect the photoresponses of isolated rods

The simplest hypothesis for the effects of CO_2–HCO_3^- and AAA would be that they somehow directly affect the phototransduction machinery in the rod outer segment. If so, the effects on single rods in isolation from the rest of the retinal tissue should be similar to the effects on rods embedded in an intact retina. We therefore investigated how current responses from an isolated rod drawn into a recording pipette with only (part of) the outer segment protruding into the perfusion fluid would be affected by bicarbonate–AAA. Rods of the tiger salamander were used

because they are much easier to record from than isolated rods of *Rana temporaria* (cf. Donner *et al.* 1989). The preparation was made in bicarbonate-free (HEPES) Ringer solution. After a rod had been found and characterized in this solution, perfusion was switched directly to a Ringer solution containing both bicarbonate and

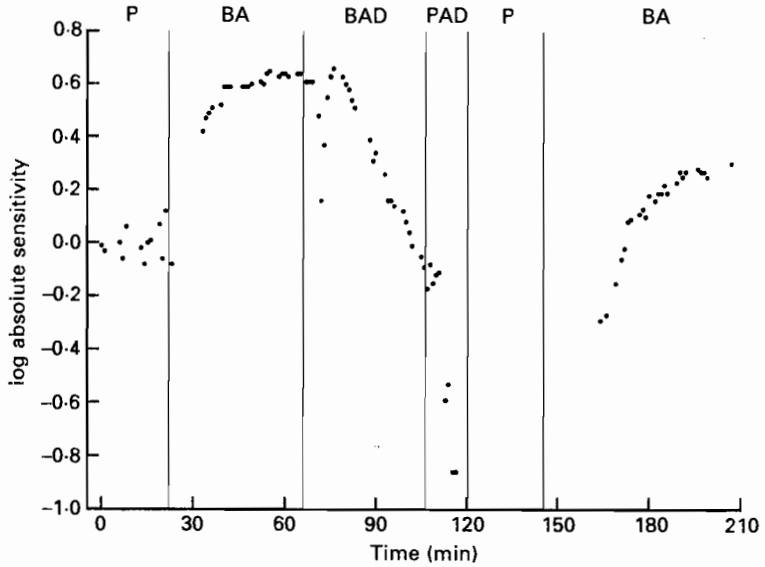

Fig. 5. The time course of changes in absolute sensitivity throughout an experiment involving the following sequence of perfusates: phosphate (P), bicarbonate–AAA (BA), bicarbonate–AAA–DIDS (BAD), phosphate–AAA–DIDS (PAD), phosphate (P) and bicarbonate–AAA (BA). The concentration of AAA was 0·5 mM and that of DIDS 0·1 mM. The ordinate gives log absolute sensitivity in relative units, where 0·0 corresponded to 0·93 μV/Rh*. Each dot, in effect, plots the amplitude of one response to a flash of intensity 6 Rh*, delivered at the time given by the abscissa.

AAA in order to bring out any possible effect with maximum force. Just-saturating flashes of light were delivered continuously at 45 s intervals throughout the critical phase of the experiments.

No effects on either the amplitude or kinetics of these responses could be discerned (experiments on four rods). This is exemplified by the responses shown in Fig. 4*A* and *B*. Panel *A* shows two saturated responses taken just before the introduction of bicarbonate–AAA, panel *B* two responses recorded after the switch, the second one at the time the effect should have been strongest. Clearly, no significantly change in photocurrent had occurred. Nor did a closer inspection of the earliest rise of the responses (not shown) reveal any changes in sensitivity or time scale. For comparison, the bottom row (Fig. 4*C* and *D*) shows a corresponding series of saturated responses from the intact retina of the tiger salamander, recorded with the same ERG technique as used in the frog experiments. The two responses recorded in phosphate Ringer solution just before the switch to bicarbonate–AAA (panel *C*) show that the saturated response amplitude was at that time slowly decreasing. The three responses recorded after the switch (panel *D*), however, show a monotonic growth over 15 min. The peak amplitude of the final saturated responses is 35% larger than that of the first, and during the early rise of the response, the amplitude difference

is 50%. Thus, the lack of effect on isolated rods is not due to species differences between the frog and the tiger salamander.

The general conclusion from these experiments is that the mechanism(s) which mediate the effects of HCO_3^- and AAA on phototransduction are not present in the

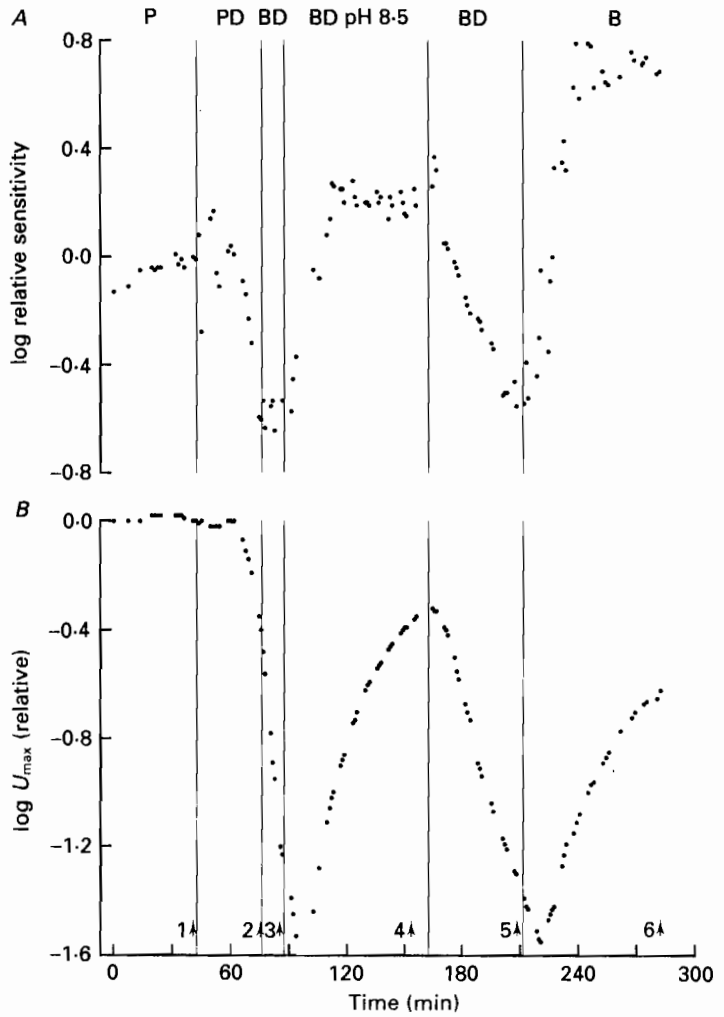

Fig. 6. The time courses of changes in log S and log U_{max} in an experiment with the following perfusate sequence: phosphate (P), phosphate–DIDS (PD), bicarbonate–DIDS (BD), bicarbonate–DIDS with pH 8·5 (BD pH 8·5), bicarbonate–DIDS (BD), bicarbonate (B). Conventions as in Fig. 2. Numbers and arrows in *B* identify responses in Fig. 7*A*.

rod outer segment. As regards AAA, the negative result may not be surprising considering that CA activity has never been demonstrated in rods (see Introduction). It is worth noting, though, that it also excludes possible side effects on the phototransduction machinery. In biochemical experiments, we checked directly the possibility of AAA effects on the light-induced hydrolysis of cyclic GMP in a suspension of rod outer segments (pH measurements; methods as described by Liebman & Evanczuk, 1982). No effects were found.

With regard to bicarbonate, it should be noted that the negative conclusion from these experiments only applies to relatively modest increases in intracellular bicarbonate. Assuming thermodynamic equilibrium and a membrane voltage of about 30 mV, CO_2 entry due to the introduction of 6 mM-CO_2–HCO_3^- will raise $[HCO_3^-]_i$ of the rod to *ca* 2 mM. It is still possible that at very high intracellular concentration HCO_3^- ions could exert some direct action on phototransduction.

Effects of DIDS

There are good reasons to think that the bicarbonate effect on phototransduction might be due to an exchange mechanism which regulates pH_i by transporting HCO_3^- into the rod (see Introduction). We therefore tested whether rod function was affected by the anion transport blocker DIDS. In different experiments, DIDS was added at different stages of the perfusion sequence, and in some experiments AAA was used together with bicarbonate.

DIDS suppresses light sensitivity completely

In Fig. 5, the amplitude of the response to a fairly dim flash (6 Rh*) is plotted throughout a typical experiment. After stabilizing in phosphate Ringer solution the retina was exposed to bicarbonate–AAA Ringer solution, which boosted dim-flash responses 4-fold. When a steady state had been reached, 0·1 mM-DIDS was added to the perfusate with dramatic effects. After a relatively long latency (about 10 min in Fig. 5, but often longer), a steep and continuous decay of photoresponses set in. The removal of bicarbonate from the perfusate (at *ca* 110 min in Fig. 5) appeared to make the decay even steeper, suggesting that the presumed HCO_3^--dependent mechanism had not been 100% inactivated. The deterioration was allowed to proceed until the rods were almost completely insensitive to light. At around 120 min in the figure, flashes delivering *ca* 10000 Rh* elicited response amplitudes that were about 1% of the pre-DIDS saturated amplitude and the responses continued to decrease.

The DIDS effect is reversible

In spite of the virtually complete loss of photoresponses, the reintroduction of bicarbonate–AAA (after DIDS had been washed out with phosphate) gradually restored photosensitivity. After 35 min in bicarbonate–AAA Ringer solution (at *ca* 180 min in Fig. 5) the size of dim-flash responses had recovered to the level that had prevailed in phosphate Ringer solution at the beginning of the experiment.

The effect of DIDS depends on pH

The experiment illustrated in Figs 6 and 7 establishes a connection between DIDS suppression and pH. Figure 6*A* and *B* traces the time courses of changes in relative sensitivity and in the amplitude of saturated responses. Figure 7*A* shows six pairs of responses to a fairly dim flash (9 Rh*) and a near-saturating flash (900 Rh*) representative of each main phase (numbered arrows in Fig. 6). Figure 7*B*, finally, shows how the kinetics of dim-flash responses changed as the pH of the perfusate was changed in the presence of DIDS.

In this experiment, DIDS was introduced into the phosphate Ringer solution, setting off a steep decay of responses, which the introduction of bicarbonate did not slow down perceptibly (Figure 6: note that the stabilizing of relative sensitivity only

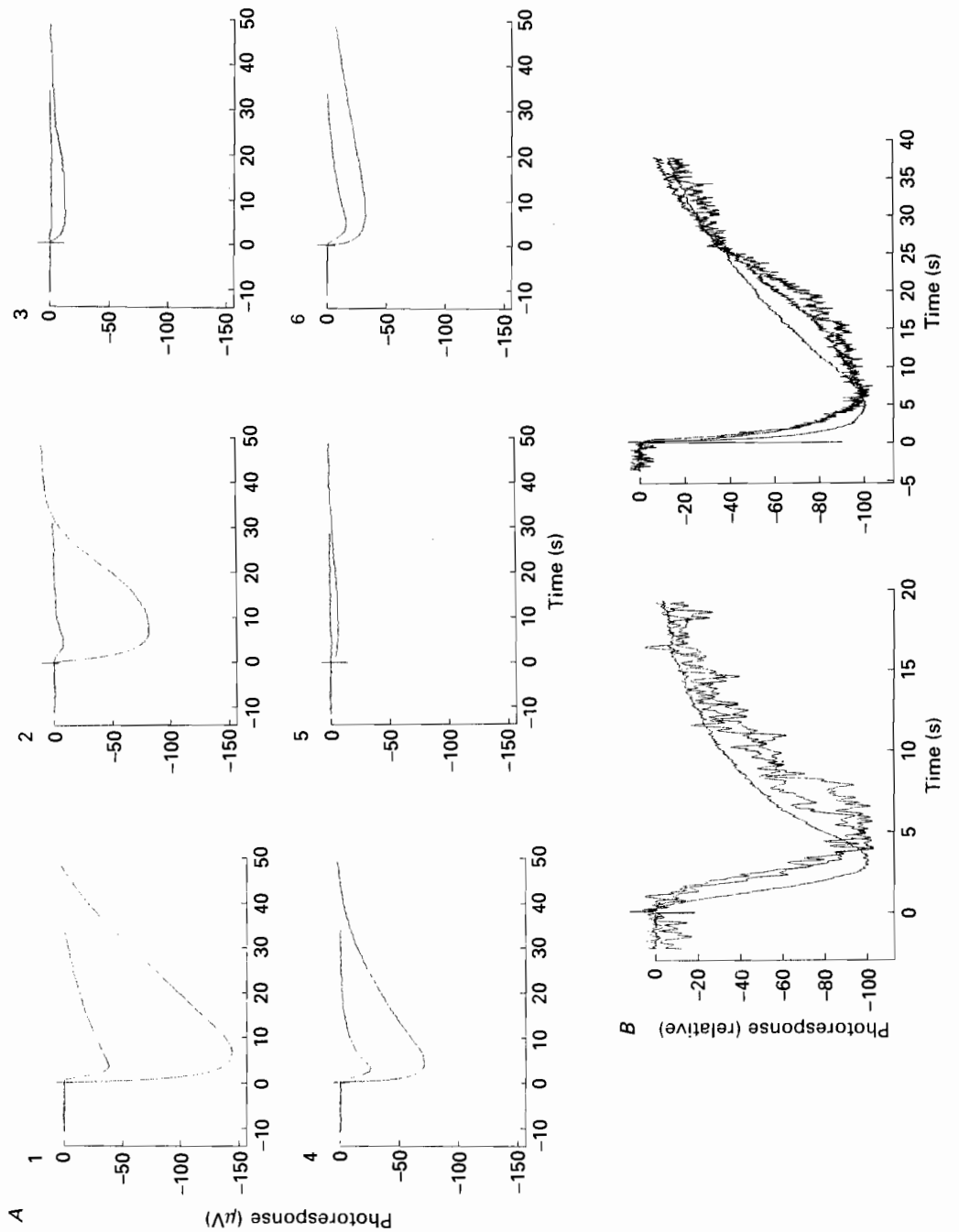

Fig. 7. For legend see facing page.

means that dim-flash responses decay at the *same* rate as saturated responses.) Then, at a stage when the saturated responses had decreased by about 95% (*ca* 90 min; responses (3) in Fig. 7), the pH of the DIDS perfusate was raised by 1 unit to 8·5. After a delay of a few minutes responses began to recover, growing monotonically for at least 1 h. At that stage the pH of the perfusion fluid was lowered back to 7·5 and almost immediately a new decay started. This was again allowed to proceed until photoresponses were almost undetectable, before DIDS was removed. A second recovery started, taking the saturating responses amplitude back to at least one-third of its initial value.

Changes in response kinetics

It is instructive to consider the kinetics of responses during the different phases of the experiment. The times-to-peak and amplitudes of the dim-flash responses shown are: 3·6 s and 38 μV (no. 1; phosphate), 4·5 s and 8 μV (2; phosphate–DIDS). 5·5 s and 1·5 μV (3; bicarbonate–DIDS), 3·0 s and 25 μV (4; bicarbonate–DIDS in pH 8·5), 4·7 s and 8 μV (6; bicarbonate). Thus, there is a fairly consistent correlation between speed and amplitude.

The time-to-peak of ERG mass responses is not an entirely reliable measure of receptor kinetics. Figure 7*B* shows that, none the less, the time-to-peak reflects the time scale of the early rise of these responses fairly well. Dim-flash responses recorded in DIDS at pH 7·5 and 8·5 are compared. It is a trivial fact that the response with the larger peak amplitude (here, the one at pH 8·5) generally rises faster in an absolute sense; however, even when scaled to the same peak amplitude, the 'alkaline' response rises more steeply than the 'acid' response (Fig. 7*B*).

The dramatic effect of changing the pH of a DIDS-perfusate stands in sharp contrast to the comparatively high resistance of the retina to pH changes in DIDS-free Ringer solution. For example, dropping the pH from 7·5 to 6·5 in phosphate or bicarbonate Ringer solutions caused only a shallow decay of photoresponses. It thus seems likely that DIDS inactivates a mechanism for the regulation of rod pH.

DISCUSSION

DIDS-sensitive HCO_3^- exchange

The complete but reversible deterioration of light sensitivity in DIDS indicates that the rods themselves possess a homeostatic mechanism which is blocked by DIDS. The return of sensitivity in elevated pH connects this mechanism with pH regulation. This, of course, does not imply that the specific pathways whereby pH

Fig. 7. *A*, response pairs taken at the times indicated by arrows in Fig. 6. The small response in each pair was elicited by 9 Rh* and the near-saturated one by 900 Rh* flashes. *B*, comparison of the waveforms of responses taken in DIDS–Ringer at pH 7·5 (noisy traces) and 8·5 (less noisy trace), scaled to equal amplitude. Left panel, responses to 9 Rh* (averages of four responses). Right panel, responses to 900 Rh*. The two noisy responses in both panels were taken before the switch to pH 8·5 and after the return to pH 7·5, respectively; these 'acid' waveforms are seen to be very similar. Relative amplitude 100 for the dim-flash responses corresponds to 23 μV ('alkaline') and 2·3 and 2·8 μV ('acid'). The amplitudes of the saturated responses were 60 μV ('alkaline') and 13 and 24 μV ('acid'), respectively.

affects phototransduction could not involve the further regulation of other ions, such as Ca^{2+}, by H^+ (cf. Pugh & Cobbs, 1986).

The presence of DIDS prevented the salutary effects on photoresponses otherwise associated with the introduction of bicarbonate. This is consistent with the idea that bicarbonate acts through a DIDS-inhibitable exchange mechanism, whose efficiency depends on the availability of extracellular HCO_3^-. While our results fit the idea that main action of bicarbonate is to prevent intracellular acidosis, we cannot exclude the possibility that strongly elevated intracellular [HCO_3^-] could also affect phototransduction in other ways, e.g. as a mobile buffer.

CO_2 transport in Müller cells and the effects of AAA

The inhibition of CA in the isolated retina creates a steady-state acidification of the inner retina (Oakley & Wen, 1989), suggesting that a system for the removal of CO_2–HCO_3^-–H^+ is impaired. In our geometry, substances can be discharged only on the receptor side of the retina, around the rod outer segments. We would then expect the inhibition of CA to cause (1) a build-up of CO_2–HCO_3^-–H^+ in the retina and (2) a relief in the H^+ load (an alkalinization) around the rod outer segments. This relief must be transient, though, because in the steady state all metabolic acid produced in the retina will pass the same way, whether or not there is active transport.

Figure 8 presents a hypothetical scheme that accounts for the results, attributing to the Müller cells a key role in the catalysed transport of retinal CO_2–HCO_3^-–H^+. This is based on two facts. Firstly, the Müller cells contain most of the CA present in the retina (see Linser & Moscona, 1984). Secondly, they are closely related to brain astrocytes which are thought to be involved in the removal of metabolic CO_2 from the vicinity of neurons discharging it elsewhere by HCO_3^-–Cl^- and Na^+–H^+ exchangers (Kimelberg, Biddlecome & Bourke, 1979; see Kimelberg & Norenberg, 1989). The assumed H^+–HCO_3^- extrusion by Müller cells would create effective outward gradients for these ions and for CO_2. The efficiency of the transport would depend on a CA-catalysed fast equilibrium in the bicarbonate buffer system. It is worth noting that it would also serve the transport of hydrogen ions originating from metabolic lactate (Winkler, 1981, 1986; Oakley & Wen, 1989).

The proposed scheme thus offers two possible causes for the growth of photoresponses when AAA is applied: (1) [HCO_3^-] increases around the (proximal?) parts of the rods where bicarbonate exchangers are located, increasing the transport of that ion into the cells; and (2) [H^+] decreases around the rod outer segments, decreasing the leakage of that ion into the cells. Both would tend to raise pH_i (and [HCO_3^-]$_i$) and we have no evidence on which to determine their relative contributions.

Correlation between response amplitude and [cyclic GMP]

The changes in photoresponses observed under bicarbonate and AAA treatments were qualitatively well correlated with changes in total cyclic GMP content (Table 1). The [cyclic GMP] increases are also in agreement with the hypothesis that bicarbonate and AAA counteract acidification of the rods. Meyertholen *et al.* (1980, 1986) found that increasing the concentrations of HEPES and/or bicarbonate buffers in a critical range between 1 and 5 mM roughly doubled [cyclic GMP] in toad retinas. Since a low buffering capacity is accompanied by retinal acidosis (Oakley &

Wen, 1989), it may be concluded that [cyclic GMP] is depressed by acidification. Interestingly, Meyertholen *et al.* (1986) found no effects of buffers on the cyclic GMP contents of isolated, incubated rod outer segments, supporting our contention that the acidification is primarily due to the acid loads accumulating in the intact retinal tissue.

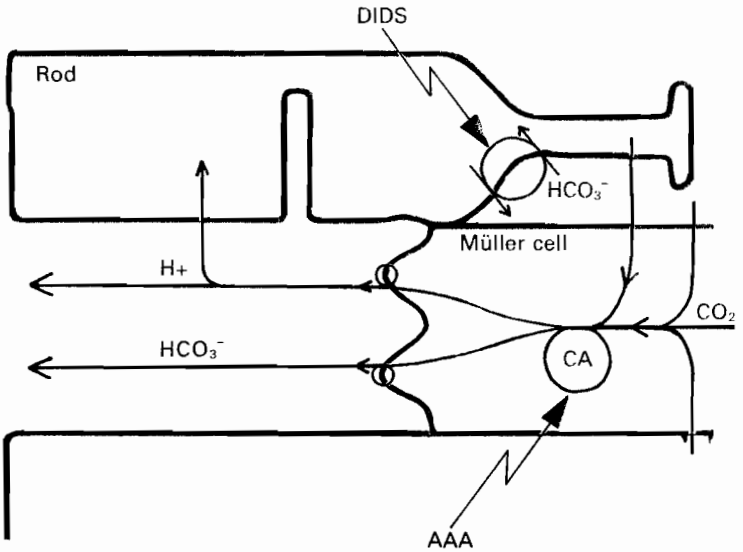

Fig. 8. A hypothetical scheme showing the relations between rods, Müller cells and other retinal cells invoked to explain the experimental results. By analogy with brain astrocytes, the Müller cell is assumed to extrude H^+ and HCO_3^- ions at one end (the scleral end). Thus, gradients arise for CO_2 into the cell, and for H^+ and HCO_3^- towards the scleral end. This Müller cell pathway out of the retina relies on the activity of carbonic anhydrase (CA) ensuring a fast equilibrium between CO_2 and HCO_3^-–H^+. Important for rod function are the H^+ ions around the outer and inner segments, which tend to leak into the rod, and the HCO_3^- ions around the DIDS-sensitive exchange mechanisms, which can be transported into the rod to counteract acidification. When CA is inhibited, the concentration of the former decreases and that of the latter increases; both changes tend to make that rod more alkaline. (In the intact eye, HCO_3^- transport across the apical membrane of pigment epithelium cells would act to neutralize the subretinal space.)

A strictly quantitative correlation between the size of the photocurrent (as expressed by the saturated response amplitude) and [cyclic GMP] is not to be expected. Firstly, the sodium conductance of the rod outer segment is determined by free cyclic GMP, while our biochemical techniques measure total cyclic GMP. Secondly, free cyclic GMP (probably) determines the sodium *conductance*, but the photocurrent can only momentarily reflect this conductance. The steady-state current is limited by the rate of sodium pumping, maintaining the driving force for Na^+. Thus, the amplitude of saturated responses would rather be expected to follow rates of change in free [cyclic GMP]. Nevertheless, the very high [cyclic GMP] value in AAA (Table 1) is remarkable in relation to the moderate growth of photoresponses. One explanation is that the retinas incubated in the biochemical experiments were floating freely in the medium. If AAA blocks the directed transport of CO_2–HCO_3^-–H^+ to the receptor side, these substances will thereafter diffuse out

equally on both sides of the retina. By contrast, in the geometry of the electrophysiological experiments, AAA can bring only a transient decrease in the acid flux around the photoreceptors (see above).

pH affects many processes in rods

In our experiments with varying pH (Figs 6 and 7), both the relative sensitivity and the saturating response amplitude, as well as the speed of responses, were increased by alkalinization and decreased by acidification. This is in partial contrast to Liebman *et al.* (1984) who found that acid perfusion brought a decrease in saturated responses but an *in*crease in relative sensitivity. However, their experiments were rather different involving short pulses (less than 1 min) of changed pH, and it is likely that pH effects on phototransduction inside the rod did not have time to develop fully.

Even our superficially similar pH effects on the saturating response amplitude may not be homologous to those of Liebman *et al.* (1984). With low pH, they found a fast dark current suppression, completed within a second, and no immediate change in the kinetics of the response rise. This has been interpreted as a retardation of the Na^+–Ca^{2+} exchange, making Ca^{2+} accumulate in the rod (Pugh & Cobbs, 1986). Our effects develop over a time scale of tens of minutes, involving changes in the kinetics of the response rise and substantial changes in total [cyclic GMP].

It is not surprising that there may be several different pH effects, since the activity of every component in the biochemical machinery of the rod is likely to have some pH dependence. The molecular mechanisms determining changes in the saturated response and the relative sensitivity are still relatively obscure. Below pH 8, phosphodiesterase activity *in vitro* decreases shallowly with decreasing pH (Miki, Baraban, Keirns, Boyce & Bitensky, 1975), which, considered in isolation, would predict that [cyclic GMP] increases with acidification. On the other hand, the pH dependence of guanylate cyclase is not known.

Conclusion

Our results are consistent with the hypothesis that rods in the intact isolated retina are relatively alkalinized by bicarbonate and AAA, and relatively acidified in bicarbonate-free perfusion. A central role is played by a mechanism which is inhibited by DIDS and is thought to transport HCO_3^- into the cell. Although the mechanisms studied here may have more to do with general homeostasis than with specific links in the transduction chain, we should like to emphasize that pH is an important determinant of the functional parameters of the rod: sensitivity and response kinetics. However, the universally reactive hydrogen ion may affect phototransduction at a great number of points and, although strong acidification undoubtedly inactivates transduction completely, there exists no monotonic dependence of these parameters on pH.

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