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Changes in the Light-Sensitive Current of Salamander Rods Upon Manipulation of Putative pH-Regulating Mechanisms in the Inner and Outer Segment

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The light-sensitive current of dark-adapted rods isolated from the *Ambystoma* retina was recorded while either the inner or the outer segment (IS or OS) protruding from the suction pipette was exposed to treatments intended to reveal the physiological roles of pH-regulating transport mechanisms. Applied to the IS, both amiloride (presumed to block Na^+/H^+ exchange, 2 mM) and 4-4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) (presumed to block bicarbonate transport, 0.1 mM) generally abolished light sensitivity completely but reversibly, consistent with acidification of the IS. Yet, the circulating ("dark") current often persisted, implying that the OS was not acidified. Applied to the OS, amiloride depressed but DIDS increased the dark current and photoresponses. Given the fact that the current increases with rising OS- pH_i , this suggests alkalization, which could be due to DIDS inhibiting bicarbonate extrusion by $\text{HCO}_3^-/\text{Cl}^-$ exchangers in the OS. Consistent with this idea, replacing external Cl^- by other anions increased the current as would be expected if $\text{HCO}_3^-/\text{Cl}^-$ exchange is reversed. We propose that the IS and OS manage their acid balances independently and with different sets of transport mechanisms. Acidosis in either compartment suppresses the photosensitivity of the rod, but by differing mechanisms.

Photoreception Retinal rods Ion exchange mechanisms Intracellular pH DIDS Amiloride
Ambystoma

INTRODUCTION

Fluorometric measurements of rod intracellular pH (pH_i) in the frog retina indicate that rods possess Na^+/H^+ exchangers, sodium-independent $\text{HCO}_3^-/\text{Cl}^-$ exchangers and (at least) a sodium-coupled HCO_3^- transport mechanism for pH_i -regulation (Kalamkarov, Pogozheva, Shevchenko, Donner, Koskelainen & Hemilä, 1994). The knowledge that certain types of pH-controlling transport mechanisms are present in rods does not as such, however, tell much about physiology, as the rod is a cell composed of distinct and highly specialized parts. The inner and outer segment (IS and OS) have completely different tasks, and their acid/base budgets are also different. Thus they are likely to have different needs with respect to H^+ transport and it appears necessary to study the function of the various types of exchangers independently in the IS and OS.

Current physiological knowledge on acid production and pH effects in rods can be summarized as follows. In the IS, energy metabolism continuously produces lactic acid and CO_2 (Winkler, 1981, 1986). In the OS, photo-transduction liberates H^+ at rates that depend on the level of light stimulation (see Liebman, Mueller & Pugh, 1984). The photoresponses and cGMP content of rods in the intact retina are depressed by treatments believed to be acidifying (Sillman, Owen & Fernandez, 1972; Gedney & Ostroy, 1978; Meyertholen, Wilson & Ostroy, 1986; Katz & Oakley, 1990; Donner, Hemilä, Kalamkarov, Koskelainen & Shevchenko, 1990). The dark current of single rods is always depressed when the OS is exposed to acidifying treatments and boosted when the OS is exposed to alkalizing treatments (Mueller & Pugh, 1983; Liebman *et al.*, 1984).

The objective of the present work is to elucidate the physiological roles of known types of pH-regulating mechanisms separately in the IS and the OS of dark-adapted rods that are in a reasonably normal state. We use suction-pipette recording of the light-sensitive current of isolated cells, which allows us to monitor changes in the physiologically relevant rod signal while selectively exposing either segment to drugs or changes

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of the ionic environment. The disadvantage is that we are left in uncertainty as to the actual pH_i displacements achieved by our manipulations. Regrettably, standard techniques for monitoring pH_i would in our case disturb the system excessively: fluorometry implies that rods are bleached, and to our knowledge, all attempts to impale retinal rods with pH-microelectrodes have been unsuccessful, essentially shunting the plasma membrane for hydrogen ions. On the other hand, it is sufficient for us here to consider only qualitatively the direction of pH_i -shifts after treatments. The monotonic relation between OS- pH_i and photocurrent that prevails at least transiently (Liebman *et al.*, 1984; and below) provides a basis for fairly firm conclusions.

METHODS

Rod current recordings

The current of isolated rods was recorded with the suction-pipette technique of Baylor, Lamb and Yau (1979) with details as described by Donner, Hemilä and Koskelainen (1989) and Donner *et al.* (1990). Dark-adapted, isolated rods of larval tiger salamanders, *Ambystoma tigrinum* (Lowrance Waterdog Farms, Tulsa, Okla, U.S.A.) or *Ambystoma mexicanum* (bred in Moscow) were used. In the present experiments we detected no significant differences between rods from these two species. The animals were decapitated and double-pithed, both eyes were enucleated and the retinas were isolated under dim red light. A piece of retina was gently torn in a small volume of Ringer to produce isolated rods, and the suspension was transferred with a syringe to the specimen chamber. Under video inspection using infra-red illumination (two Schott RG850 long-pass filters in the illuminating beam), the outer or inner segment was drawn into the tip of a fire-polished silanized glass micropipette.

In this paper we consider changes in (1) the *dark current*, which is the total current flowing from the inner to the outer segment when the rod is not stimulated by light; (2) chiefly, the *light-sensitive current* or *photocurrent*, which is that part of the dark current that can be turned off by light, and can be readily measured as the amplitude of photoresponses to saturating light stimuli. Usually, the two are the same, but when the IS was treated with amiloride or 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) this was not always the case (see Fig. 2). The intensities of the 50 msec, 494 nm full-field stimuli were adjusted to be just saturating in each experimental condition, i.e. to about 50 times the intensity needed to produce a half-maximal response. Sensitivity was regularly monitored with weaker flashes and the intensity of the flashes meant to be just-saturating was adjusted if necessary. The signal from the current-to-voltage converter was digitized and stored on a computer hard disk for off-line analysis. The experiments were done at 21°C.

Solutions and perfusion

The Ringer solutions contained (mM): NaCl 95, KCl 3, CaCl_2 0.9, MgCl_2 0.5, glucose 10, and pH buffers 12. The buffer was either 12 mM sodium-HEPES buffer (*HEPES Ringer*), or 6 mM sodium bicarbonate plus 6 mM sodium-HEPES (*bicarbonate Ringer*). For perfusion we used 95% Ringer plus 5% Leibovitz culture medium L-15 (Sigma), which improved the viability of the rods. L-15 contains phenol red, but at the low concentration and the high stimulus intensities involved, the light absorption of this substance was of no importance. pH was adjusted (by adding NaOH or HCl) to 7.5 unless otherwise noted.

The Ringers were always prepared just before the experiment. It was not then necessary to stabilize solutions containing 6 mM bicarbonate by gassing. CO_2 escapes very slowly at such low bicarbonate concentration and high pH, so with 6 mM HEPES present, the solutions are stable enough with respect to both pH and $\text{CO}_2\text{-HCO}_3^-$. This was checked by pH measurements in two Ringers (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 solution. Every 45 min pH was measured and the solutions thoroughly stirred. (Normally solutions were, of course, not stirred after preparation, and the portions used in the experiment were taken with a syringe from the bottom of the beaker.) Small alkalizations were observed, increasing approximately linearly over time and amounting after 6 hr to 0.1 pH units in the bicarbonate-HEPES solution and 0.3 pH units in the Ringer buffered with 12 mM bicarbonate alone. The former value provides an upper limit to the possible inaccuracy of pH_o in our experiments (usually lasting no more than 3 hr). The latter value indicates that the relative change in the bicarbonate concentration of our solutions was negligible. We are confident that CO_2 escaping from 6 mM HEPES + 6 mM bicarbonate Ringers was not a significant source of error.

In some experiments 95 mM of sodium chloride in the Ringer was substituted by sodium gluconate. Because gluconate binds calcium (Skibsted & Kilde, 1972), 2.37 mM extra CaCl_2 was added to the gluconate Ringer to keep free $[\text{Ca}^{2+}]$ constant. The required addition of Ca^{2+} was calibrated by titration in bicarbonate Ringer at pH = 7.5 with a Ca^{2+} -sensitive electrode (Orion Research Corp. model 93-20). In the experiments where 95 mM chloride was substituted by methylsulphonate there was no need to adjust Ca^{2+} .

Low- Ca^{2+} solutions contained 50 μM Ca^{2+} and 195 μM EGTA, giving a final concentration of 10 nM free Ca^{2+} .

The chemicals (Sigma) employed to affect acid-base transport were: (1) DIDS (0.1 mM); (2) amiloride (2 mM); (3) carbonylcyanide-*m*-chlorophenylhydrazine (CCCP) (20 μM), a protonophore which was used to make membranes leaky to H^+ ; (4) monensin (0.1 μM), an artificial Na^+/H^+ exchanger.

In our gravity-driven perfusion system, one solution replaced another in about half a minute after a switch.

RESULTS

The results to be presented document changes in rod current, which are *interpreted* in terms of pH_i and the different physiology of two separate compartments, the inner and outer segment (IS and OS). The logic will be easier to follow if one main *basis of interpretation* is kept in mind continuously: rod dark current and photocurrent always decrease when hydrogen ions are introduced into the OS and increase when hydrogen ions are removed from the OS (Liebman *et al.*, 1984; see Fig. 4). Thus, for example, if current does not change, OS- pH_i cannot have changed.

The inner segment

Effect of amiloride. To study the role of Na^+/H^+ exchange, we applied the inhibitor amiloride (2 mM). The OS was drawn well past the pipette constriction to be protected from the drug. Amiloride always depressed photoreponses, but its effectiveness varied substantially between rods. The underlying differences in the physiological state of the cells appeared to correlate with the initial size of the light-sensitive current. In all experiments where 2 mM amiloride was applied alone to the IS of a rod initially giving saturated responses of reasonably large amplitude (25 pA or more), it set off a decay which ended in complete and irreversible loss of light sensitivity within *ca* 10 min (in one cell 20 min). This was true even in bicarbonate Ringer (containing 6 mM

FIGURE 1. Changes in the saturated flash response of a rod when the IS was perfused with 2 mM amiloride. (A) Bicarbonate Ringer, (B) bicarbonate-buffered low- Ca^{2+} solution. Each dot plots the amplitude of a saturated photoreponse recorded at the time indicated by the abscissa. Time of amiloride application indicated by vertical line. The lower panels display representative photoreponses recorded at the times indicated by the correspondingly numbered arrows.

FIGURE 2. Persistence of dark current after loss of photosensitivity in IS-perfusion with 2 mM amiloride. (A) Changes in the recorded current as a rod was moved back and forth in the pipette after photoresponses had completely disappeared, recorded in HEPES Ringer without amiloride. The record starts with the rod wholly on the outside of the pipette constriction (left) and ends with the whole rod inside the pipette (right); in either case there is of course no current flowing through the recording system. Other important positions are marked with numbered arrows: (1) almost the whole OS inside the pipette, i.e. the cilium just outside the constriction; (2) almost the whole rod again blown outside of the constriction; (3) the cilium is just passing the constriction as the rod is being drawn back into the pipette, i.e. the whole OS is inside. The saturated response amplitude at the beginning of the experiment (before drug application) was 32 pA. (B) Continuous recording around amiloride application at time 0 from another rod where the decrease in response amplitude involved both decreasing dark current and an increasing proportion of light-insensitive current. The record shows absolute d.c.-levels throughout (no shifts), and although the first five responses were recorded as separate, they are also correctly placed on the time axis. The growing gap between the response saturation level and the zero current level (dotted line) from *ca* 3 min after drug application onwards indicates how more and more of the circulating current becomes insensitive to light.

HCO_3^-). The results in Fig. 1(A) are representative of such rods ($n = 10$). In rods where the initial light-sensitive current was small (*ca* 20 pA or less), however, the effect developed only over a long time in amiloride perfusion, even 1 hr or more ($n = 5$). A tentative explanation is that resilience to amiloride might be connected with low metabolism (low acid production) in cells with small circulating current (cf. similar DIDS results below).

Amiloride is not very specific (see below) and conceivably, effects could be due to inhibition of $\text{Na}^+/\text{Ca}^{2+}$ rather than Na^+/H^+ exchange, or to other interference with intracellular calcium. To investigate this possibility we applied the drug also in low- Ca^{2+} perfusion (see Methods). However, there was little to distinguish these results [three experiments, exemplified in Fig. 1(B)] from those in normal calcium. Thus, we find no evidence that amiloride effects in the IS depend on inhibition of $\text{Na}^+/\text{Ca}^{2+}$ exchange.

Persisting dark current after loss of photosensitivity. A most interesting finding regarding the IS-action of amiloride (or DIDS, below) was that even when photoresponses had completely disappeared, the circulating (dark) current would partly or even almost wholly persist. This is an important clue to mechanism, because

when the *outer segment* is acidified, the dark current itself is suppressed (Liebman *et al.*, 1984).

Our main body of evidence is from experiments where rods that had already lost their photosensitivity completely were moved back and forth in the pipette [exemplified in Fig. 2(A)]. If a circulating current remains, changing the position of the rod will change the current fraction that passes through the recording system. Thus the current can be measured independently of photoresponses. In the experiment in Fig. 2(A), the persisting dark current was nearly undiminished compared with the original photocurrent.

Such recordings are in principle sensitive to pressure artifacts that may be connected with moving the rod in the pipette. This cannot, however, explain our observations. First, the pressures applied are transient in our system. When we stop blowing or sucking, pressure evens out with a time constant of *ca* 5 sec and thus cannot cause sustained differences in apparent current levels. Second, we never observed sustained changes of level when moving current-less rods (or rod outer segments) as often happens when a preparation is scanned for a good cell. Third, when the rod was moving inwards under continuous suction [e.g. Fig. 2(A), arrow 3], the recorded signal rose to a maximum, then fell as the

cilium passed the pipette constriction, although there were no correlating pressure changes.

Persisting dark current was observed only in those rods where amiloride had abolished photosensitivity comparatively quickly. In cells where amiloride effects developed slowly (over up to 1 hr), the dark current disappeared together with photosensitivity.

Figure 2(B) illustrates results from another type of experiment, where the circulating current and saturating photoresponses were continuously recorded around the time of drug application. If the baseline is stable enough, so the zero current level is reliably known, it is then possible to continuously differentiate between decreases in dark current on one hand and failure of saturating flashes to extinguish the current on the other hand. This type of experiment was done on five cells. Two were cells where responses decreased very slowly (see above) and these did not reveal any light-insensitive current. In the three others, various proportions of the original dark current persisted, and the estimates were consistent with those from moving the same cell in the pipette after the complete disappearance of photoresponses. In Fig. 2(B), amiloride (applied at time 0) almost immediately caused a dark current decrease, but from *ca* 3 min onwards the peaks of saturated response also fall increasingly short of the zero level, indicating that a successively larger proportion of the current is becoming insensitive to light. This record is discontinued at about 13 min after drug application because then there appeared more baseline instability and we could no longer be sufficiently sure of the real zero current level.

Effect of DIDS. To study the role of HCO_3^- transport, we applied the inhibitor DIDS (0.1 mM) in bicarbonate-containing Ringer. The results were essentially similar to those with amiloride, although there were generally longer delays before DIDS produced visible effects. In rods with reasonably large initial current, perfusion of the IS with DIDS always caused a thorough decay of photoresponses within 10–30 min (Fig. 3, representative of six experiments). This was preceded by a considerable but highly variable (a few minutes up to half-an-hour) latent period. The degree of reversibility depended on how soon DIDS was washed out: if responses were allowed to drop very strongly before DIDS was removed, the effect was irreversible, ending either in complete loss of light sensitivity or a permanent depression of the saturated response to a very low level (1–3 pA). Otherwise it was largely reversible. Like in amiloride, we observed (by moving the rod in the pipette) that dark current often remained even when photoresponses had more or less completely disappeared (observations in three cells, not shown).

In the experiment of Fig. 3, 2 mM amiloride was applied after DIDS had been washed off. The effect of amiloride was modest in this situation, consisting only of a transient drop by about 50% in the saturated response amplitude. Within 10 min, responses recovered almost to the pre-amiloride level even with the drug continuously present. This supports the notion that the relative importance of different mechanisms for acid–base trans-

port in the IS much depends on the physiological state of the cells.

Indeed, physiological variation between cells similar to that seen with amiloride was apparent also with DIDS. In two rods with small (<20 pA) initial light-sensitive currents, IS-perfusion with 0.2 mM DIDS caused a decrease by only *ca* 50% in the amplitudes of saturated photoresponses over 1 hr.

Lack of effect of changing external pH. Changing pH_o around the IS in the range 7.1–7.8 had no effect on the light-sensitive current (two cells). If it is accepted that the effects of amiloride and DIDS are mediated by drops of pH_i (see Discussion), the lack of effects of changing pH_o may be interpreted in two ways. Either the efficiency of the pH-controlling mechanisms in the IS is powerfully regulated, or the IS-functions essential for phototransduction are depressed only by very strong acidifications.

Lack of effect of substitution or external chloride. Substitution of external chloride has been found to alkalinize frog rods, presumably due to $\text{HCO}_3^-/\text{Cl}^-$ exchange, which under strongly outward Cl^- gradients will drive in bicarbonate at a high rate (Kalamkarov *et al.*, 1994). To study the possible role of such a mechanism in the IS, we replaced 95 mM NaCl by equimolar Na-gluconate (keeping free Ca^{2+} constant, see Methods). The result was a transient decrease of the light-sensitive current (range 14–21%, $n = 4$) typically peaking 4 min after the switch. This might be due to rod depolarization as Cl^- leaves through chloride channels (Bader, Bertrand & Schwartz, 1982). With respect to pH, however, the result indicates either that there exist no chloride-dependent bicarbonate exchangers in the IS, or that alkalinization above the physiological value at least does not improve IS-functions relevant to phototransduction (see Discussion).

Note that the lack of effects both of pH_o -changes and of chloride substitution around the IS clearly contrasts with the results obtained in the OS (below). A general conclusion is that *if* either treatment did produce changes in IS- pH_i , the changes cannot have spread to the OS.

The outer segment: pH-dependence of the current

As pointed out earlier, our interpretations depend critically on a monotonical relation between the light-sensitive current and pH_i in the range with which we are concerned (Mueller & Pugh, 1983; Liebman *et al.*, 1984). We shall therefore first consider some limits to that relation.

Transient effect of moderate changes in pH_o . Liebman *et al.* (1984) showed that lowering pH_o around the OS reduces the dark current within 1 sec. They presented evidence (current decrease after introduction of weak acids or NH_4Cl wash-out) that the action was at least partly through pH_i . A general quantitative relation even between external pH and current cannot be derived from their experiments, however, because of the brief periods (a few tens of seconds) their rods were exposed to test pH_o . Thus, they achieved complete dark current

FIGURE 3. (A) Time-course of changes in the saturated response amplitude of a rod when the IS was perfused with DIDS (0.1 mM) and thereafter amiloride (2 mM). Horizontal bars indicate intervals of drug exposure. Bicarbonate Ringer. (B,C) Representative photoresponses from the various stages of the experiment, corresponding to the numbered arrows in (A).

suppression only at $\text{pH}_o = 3.5$, while in our experience rods require pH_o more than 2 units higher to retain photosensitivity in continuous perfusion.

They also failed to observe that the effect of moderate pH_o changes (range 7–8) on the light-sensitive current is essentially transient. Figure 4(A) shows changes in the saturated response amplitude in an experiment where the OS was perfused with bicarbonate Ringer first at pH 7.5, then 7.2 and finally 7.8. The fact that the light-sensitive current always returned to the original level indicates the presence of feedback regulation (note particularly the transitory underswing in pH 7.8). As indexes of pH_i -

changes after manipulation of transport mechanisms, the transient amplitude changes are obviously most useful.

If pH_i -regulation is circumvented, however, a monotonical relation is apparent even between *steady-state* response amplitude and pH_o , as exemplified in Fig. 4(B). Here, the protonophore CCCP ($20 \mu\text{M}$) was applied to the protruding OS to increase its H^+ permeability, implying that pH_i could be clamped more firmly by means of pH_o (this of course does *not* mean that pH_i becomes equal to pH_o). The responses were recorded only when amplitudes had stabilized to a

FIGURE 4. (A) Time-course of changes in the saturated response amplitude of a rod when the OS was perfused at pH_o 7.5, 7.2 and 7.8. Bicarbonate Ringer. Vertical lines indicate times of pH_o changes. (B) Saturated flash responses of another rod recorded while the OS was perfused with $20 \mu\text{M}$ -CCCP Ringer at different pH_o values (indicated beside the responses). Each trace is the average of three responses recorded in the steady state, except the 7.5 trace which is the average of two responses recorded while responses were still decaying. At pH 7.5 photoresponses finally became non-recordable, but returned when pH was raised again.

steady level after each pH switch. Although there is an overall depression of the saturated response amplitude in CCCP, a monotonical pH-dependence emerges. Even in these experiments, however, it is not possible to calculate pH_i , because pH near the cell surface (on which the electrochemical equilibrium across the membrane will depend) will be more acidic than the nominal pH of the Ringer (due to H^+ extrusion, surface charges and unstirred solution layers).

Effect of amiloride on the outer segment

Amiloride when applied to the OS suppressed the light-sensitive current significantly (by 75% on average in four rods). Drug wash-out restored a small proportion, typically bringing the response amplitude back to about half its initial value. In the experiment illustrated in Fig. 5, a weak background light was shining, the purpose being to increase acid pressure by increasing the rate of H^+ liberation from cGMP hydrolysis. Indeed, the amiloride effect was comparatively large in this experiment.

The depression of the light-sensitive current is obviously consistent with acidification due to blocking of Na^+/H^+ exchange. However, as the amiloride block of Na^+/H^+ exchangers is usually reversible (Benos, 1988), we think that at least the irreversible part of the effect is not related to pH. Amiloride and its derivatives are generally not specific and certain "side" effects are likely to be grave when the drugs are applied to the rod OS [blocking of some sodium channels, inhibition of $\text{Na}^+/\text{Ca}^{2+}$ exchange (Benos, 1988; Nicol, Schnetkamp, Saimi, Cragoe & Bownds, 1987)].

Bicarbonate transport in the outer segment

DIDS increases the light-sensitive current. When applied to the OS, DIDS always increased the photocurrent (mean 18%, range 9–25% in seven cells, see Fig. 7). This is consistent with inhibition of sodium-independent $\text{HCO}_3^-/\text{Cl}^-$ exchangers (Kalamkarov *et al.*, 1994): given that Na^+/H^+ exchange in the OS keeps pH_i above thermodynamic equilibrium, such exchangers would normally extrude bicarbonate and thus act to acidify

FIGURE 5. Time-course of changes in the saturated response amplitude of a rod when the OS was perfused with 2 mM amiloride (time of application indicated by vertical line). The variability of the photocurrent that is evident particularly before drug application is due to the presence of a weak background light (see text).

FIGURE 6. Chloride substitution around the OS boosts photoresponses. (A) Time-course of changes in the saturated response amplitude when 95 mM NaCl was replaced by equimolar Na-gluconate at constant free calcium. (B) Representative photoresponses recorded at the times indicated by numbered arrows in (A).

the cell. This hypothesis was tested in two types of experiments.

Experiment 1: removal of Cl⁻ around the outer segment boosts photoresponses. The direction of transport of the presumed HCO₃⁻/Cl⁻ exchangers should be reversed (making bicarbonate enter the cell) if [Cl⁻]_o is substantially lowered (see above). The ensuing alkalization should increase the light-sensitive current. Indeed, when 95 mM NaCl was replaced by equimolar Na-gluconate (at constant free [Ca²⁺], see Methods), this always increased the saturated response amplitude (mean 26%, range 10–53% in 14 cells). Figure 6 shows the time-course of amplitude changes and representative responses from one experiment. The results of three experiments where Cl⁻ was replaced by methylsulphonate instead of gluconate were entirely consistent (mean increase 23%).

If the chloride substitution effect is mediated by HCO₃⁻/Cl⁻ exchangers, it should be attenuated by DIDS. The results of experiments to test this were somewhat difficult to interpret, however. DIDS at 0.1 mM concentration caused no clear change and only at 1 mM concentration, DIDS consistently attenuated the effect. Figure 7 shows typical results involving effects of Cl⁻-substitution and DIDS-application separately and jointly. In this experiment, the mean response growth upon Cl⁻ replacement was 27% without DIDS and 13% in the presence of DIDS. The interpretational difficulty arises from the fact that DIDS itself already kept response amplitudes on an elevated level. It might then be argued that the weakened effect of Cl⁻ substitution is to an unknown degree due to saturation of the growth capacity rather than to inhibition of the chloride-dependent mechanism.

Experiment 2: DIDS boosts photoresponses strongly in the presence of monensin. The hypothesized bicarbonate

extrusion from the OS by HCO₃⁻/Cl⁻ exchangers should be accelerated if H⁺ extrusion by other mechanisms is enhanced. To achieve this, we introduced the artificial Na⁺/H⁺ exchanger monensin (0.1 μM) into the OS plasma membrane. This pretreatment has the further advantage that possible down-regulation of native Na⁺/H⁺ exchangers, which would damp incipient alkalizations (thus obscure the DIDS effect), is partly bypassed. The prediction—DIDS should augment responses particularly strongly in the presence of monensin—was borne out by the experiments, as illustrated in Fig. 8.

The initial introduction of monensin transiently increased the saturated response amplitude, consistent with alkalization due to amplified Na⁺/H⁺ exchange. The subsequent elevation of pH_o to 7.7 confirmed that the rod was still in a state where responses grew with rising pH. After return to pH_o = 7.5, DIDS was introduced, boosting the saturated response by no less than 80%. Again, however, the effect was transient, and the final DIDS wash-out was associated with only a minor response decrease. In four such experiments, DIDS induced a mean amplitude increase of 57% (in the same experiments, the mean increase caused by raising pH_o from 7.5 to 7.7 was 46%). This is significantly larger than the average increase due to DIDS without monensin (18%, see above). Although the numbers should be judged with some caution in view of the physiological variation that exists between cells, the results provide additional support to the idea that DIDS inhibits a mechanism that acts to acidify the outer segment. However, they also emphasize that, on a somewhat longer time scale, feed-back mechanisms either keep pH_i constant, or keep the light-sensitive current constant despite changes in pH_i.

FIGURE 7. (A) Effect of 1 mM DIDS on the "chloride substitution effect", i.e. on the changes in saturated response amplitude induced by replacing 95 mM NaCl by Na-glucuronate. Also seen is the effect of DIDS in itself (at 15–20 min). The perfusion sequence is indicated by horizontal bars beneath the record. The basic solution was bicarbonate Ringer. (B) Representative photoreponses recorded at the times indicated by numbered arrows in (A).

DISCUSSION

Rod current and pH in the inner and outer segment

The observed changes of rod current are interpreted in terms of changes in pH_i. In a wide range of cell types DIDS and amiloride have been found to block HCO₃⁻ transport and Na⁺/H⁺ exchange, respectively, and rods in the intact frog retina are acidified by these drugs (Kalamkarov *et al.*, 1994). Thus, there are strong grounds to assume that the decay of photoreponses when the IS is exposed to amiloride or DIDS is due to acidification (see further below). As regards DIDS, this is almost an inescapable conclusion: the present results show that the acidification observed in frog rods cannot have arisen in the OS, hence it must have arisen in the IS. For the OS, our interpretations are based on the fact that the dark current and saturated response amplitude grow with rising pH_i in a certain range (Liebman *et al.*, 1984; and Fig. 4).

Compartmentalization of the rod with respect to pH

Under many treatments the IS and the OS behaved differently, and DIDS even had opposite effects depending on which segment was exposed. We think that the results are best understood on the assumption that, from the viewpoint of pH_i regulation, the OS and IS form independent compartments, separated by a diffusion barrier against pH_i equilibration and having different sets of ion transport mechanisms. A barrier against pH_i equilibration is *a priori* likely from some general properties of the rod: the narrowness of the passages between the disks and the plasma membrane, the high content of non-mobile buffers especially in the outer segment

FIGURE 8. Perfusion of the OS with DIDS increases the light-sensitive current strongly in the presence of 100 nM monensin. The time-course of changes in the amplitude of saturated photoresponses in an experiment involving the perfusate sequence indicated by the horizontal bars.

(Dearry, 1981), and low $[H^+]_i$ (assuming pH_i around 7), all in relation to a relatively high H^+ turnover in either segment independently (Appendix). A rough calculation indicates that longitudinal flow of H^+ as such is orders of magnitude below H^+ production in either segment of a functioning rod (admittedly, mobile buffers at unknown concentration could in principle significantly increase acid movement). The following discussion develops the idea that the two segments form separate compartments.

Inner segment

Acidification of the IS. In the IS, energy metabolism is a continuous source of acid. It is likely that the primary action of both amiloride and DIDS is to impede extrusion of this acid and thereby make IS- pH_i acidic. The experiments with amiloride in low Ca^{2+} exclude the possibility that amiloride in the IS mainly acts through $[Ca^{2+}]_i$ rather than pH_i . The notion that the acidification is confined to the IS is supported by several results.

- (1) The dark current could persist nearly undiminished when photosensitivity had been completely abolished by amiloride or DIDS. Had the underlying acidification spread to the OS, the dark current would necessarily have been suppressed.
- (2) When pH_o outside the IS was changed, at least a transient change in IS- pH_i is likely to have occurred. Since photoresponses were unaffected, there were apparently no corresponding pH_i -changes in the OS.
- (3) Effects on phototransduction when amiloride or DIDS was applied to the IS often became evident only after substantial delays. If the underlying acidifications had been "seen" directly by the transduction machinery in the OS, effects should

have appeared more or less immediately (Liebman *et al.*, 1984; Figs 4 and 8).

Consequences of IS-acidification. Both amiloride and DIDS applied to the IS caused a monotonic but comparatively slow (time scale tens of minutes) deterioration of photoresponses. One link in the causation chain is probably the depression of energy metabolism in the IS, which is known to be one consequence of acidification (Winkler, 1986). Subsequent depletion of, e.g. GTP and/or ATP in the OS could have mediated the effect on phototransduction (suppression of transducin activation and rhodopsin phosphorylation, etc.). At least in the cases where the dark current persisted, cGMP obviously cannot have been the "minimum factor". A corollary would be that transducin activation requires a higher GTP concentration than the guanylate cyclase.

The occasional persistence of nearly undiminished dark current also implies that the sodium gradient was little affected. This excludes an alternative explanation of the disappearance of photoresponses in DIDS: the critical action cannot have been inhibition of the Na^+ , K^+ -ATPase (Pedemonte & Kaplan, 1988).

Transport mechanisms. The thorough suppression of photosensitivity that could be achieved both by DIDS alone and by amiloride alone indicates that both bicarbonate transport and Na^+/H^+ exchange are important for maintaining pH_i in the IS. It seems somewhat remarkable that blocking *either* of these mechanisms should suffice to acidify the IS, but the large variation between rods precludes conclusions regarding the relative physiological importance of the two classes of mechanisms. The activity of exchangers is generally thought to be strongly regulated. In our case, relative activities may vary depending on variations not only in general metabolic level, but also e.g. in the proportions of aerobic vs anaerobic metabolism and consequently the proportions of lactic acid and CO_2 produced (Winkler, 1981, 1986). The fact that the IS in some conditions was able to maintain physiological pH_i even in the continued presence of amiloride (e.g. Fig. 3) is consistent with the finding that 2 mM amiloride modifies but does not abolish rod photoresponses in the intact, perfused retina of the toad (Katz & Oakley, 1990) or the frog (our unpublished experiments). In the intact tissue HCO_3^- -import could be relatively more potent than in our experiments owing to higher extracellular bicarbonate concentration. Arterial $[HCO_3^-]$ in *Ambystoma tigrinum* at 21°C lies around 20–25 mM (see Toews & Boutilier, 1986), about four times higher than in our bicarbonate Ringer.

The fact that DIDS always acted as if acidifying the IS suggests that the presumed bicarbonate-transporting mechanism in the IS is coupled to the sodium gradient (contrary to the situation in the OS, see below). The finding that chloride substitution around the IS did not augment the light-sensitive current might be construed as evidence for a sodium-bicarbonate coporter (see e.g. Hughes, Adorante & Miller, 1990). However, it is also consistent with a bicarbonate/chloride exchange

mechanism, if it is assumed that IS-functions relevant to phototransduction cannot be improved by raising pH_i above the prevailing "physiological" level.

Outer segment

In the OS, H^+ liberated by synthesis and hydrolysis of cGMP constitutes the main acid load. The reutilization of the same amount of H^+ in the IS is assumed not to relieve this load (see Appendix). The dark current is known to react rapidly to changes of pH in or around the OS, being completely suppressed by strong acidification and increasing at least transiently with alkalization in a physiological range (see above).

Bicarbonate exchange. The response growth in DIDS would thus be interpreted to reflect OS-alkalinization. The idea that this was due to inhibition of sodium-independent HCO_3^-/Cl^- exchangers that are normally driving HCO_3^- out of the OS was supported by the fact that responses also grew upon lowering of $[Cl^-]_o$, a treatment expected to reverse the direction of HCO_3^- transport (Kalamkarov *et al.*, 1994). Although the fact that DIDS did not very effectively block this "chloride substitution effect" (Fig. 7) appears to go against this idea, two points should be noted. Firstly, replacement of 95 mM external Cl^- will create a very strong outward chloride gradient, allowing even rather an insignificant proportion of non-blocked HCO_3^-/Cl^- exchangers to carry a substantial inward HCO_3^- flux. Secondly, the pH-dependence of the rod current is rather steep (Figs 4 and 8).

The fact that DIDS applied to the OS never reduced the light-sensitive current even transiently argues against the presence (in the OS) of an additional, sodium-coupled mechanism driving bicarbonate into the cell. Inhibition of such a mechanism would necessarily be acidifying.

Na^+/H^+ exchange. If blocking HCO_3^-/Cl^- exchange by DIDS alkalinizes the OS, this implies that pH_i is normally kept above thermodynamic equilibrium by a sodium-coupled mechanism. The most likely candidate is the Na^+/H^+ exchanger. The response reduction when amiloride was applied to the OS is consistent with this, even though a large fraction of that effect may be unrelated to pH.

Mechanisms of pH-action on photoresponses. The phototransduction machinery is directly exposed to OS- pH_i . The mechanisms whereby the light-sensitive current is affected are beyond the scope of this paper, but we wish to emphasize that there are many possible mechanisms acting on different time-scales and in different pH ranges. Two fairly direct and rapid effects have been characterized: (1) Na^+/Ca^{2+} exchange is weakened by lowered pH (Hodgkin & Nunn, 1987), leading to rising intracellular Ca^{2+} and thus a decrease in the light-sensitive current (see also Pugh & Cobbs, 1986); (2) the Na^+ conductance of inside-out patches of outer-segment plasma membrane is halved at $pH \approx 5.5$ compared with neutral perfusion (Menini, 1989). Little is at present known about the pH-dependence of the phototransduc-

tion reactions or, for example, H^+-Ca^{2+} interactions other than through the Na^+/Ca^{2+} exchanger.

Functional role of Na^+/H^+ and HCO_3^-/Cl^- exchangers in the outer segment. The combination of Na^+/H^+ exchangers and HCO_3^-/Cl^- exchangers in the same membrane has been assumed to serve either pH_i homeostasis or the control of cell volume depending on the situation (discussed by Cala & Grinstein, 1988). From the viewpoint of pH, these exchangers normally work in opposite directions, Na^+/H^+ exchange driving pH_i above thermodynamic equilibrium and HCO_3^-/Cl^- exchange striving to restore it (e.g. Olsnes, Ludt, Tonnesen & Sandvig, 1987). In the rod OS, such an opponent system might be useful to ensure homeostasis in the face of alkalinizations and acidifications associated with the transduction and transmission of light signals (Oakley & Wen, 1989; Borgula, Karwoski & Steinberg, 1989). On the other hand, controlled changes of pH_i , as could be sensitively provided by a combination of opposing, regulated exchangers, might also be used for modulating several aspects of phototransduction.

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APPENDIX

Estimation of Acid Production

The major sources of acid in rods are cGMP metabolism in the OS and energy metabolism in the IS. We assume that the outer and inner segment can be considered as independent compartments. Further, it should be noted that H^+ leakage through the light-sensitive channels and the plasma membrane is negligible (see Kalamkarov *et al.*, 1994).

Outer segment

The main processes of interest are cyclic GMP synthesis by guanylate cyclase and its hydrolysis by phosphodiesterase. If one hydrogen ion is released in both reactions (see Goldberg, Ames, Gander & Walseth, 1983), two hydrogen ions are released into the cytoplasm per molecule of cyclic GMP. Pugh and Lamb (1990) have estimated that the dark rate of cyclic GMP hydrolysis is $2.5\text{--}3\text{ }\mu\text{M sec}^{-1}$ (cytoplasmic concentration) on the basis of biochemical (Dawis, Graeff, Heyman, Walseth & Goldberg, 1988) and electrophysiological (Hodgkin & Nunn, 1988) measurements, giving $5\text{--}6\text{ }\mu\text{M-H}^+\text{ sec}^{-1}$ in darkness. In intense light, the rate of cyclic GMP hydrolysis is increased about tenfold (Dawis *et al.*, 1988; Hodgkin & Nunn, 1988), giving approx. $50\text{ }\mu\text{M-H}^+\text{ sec}^{-1}$.

Inner segment

Metabolic acid arises as lactic acid from glycolysis and CO_2 from oxidative metabolism. A significant part of the ATP is produced by anaerobic glycolysis (Winkler, 1981, 1986), and indeed, rods in the intact retina continue to respond very well for long periods in nearly oxygen-free perfusion (Donner, Hemilä & Koskelainen, 1987). Here we estimate the maximum acid load in the two extreme cases that (1) all ATP is produced by oxidative metabolism, (2) all ATP is produced by anaerobic glycolysis.

The dark current of salamander rods as used here is about 80 pA, corresponding to $83 \times 10^{-17}\text{ mol sec}^{-1}$. Assuming that in darkness most ATP is consumed by the Na^+, K^+ -ATPase where 1 ATP molecule is used to drive three Na^+ ions out, we get an estimate of $28 \times 10^{-17}\text{ mol-ATP consumed per second}$.

(1) By oxidative metabolism, 36 ATP and 6 CO_2 molecules are generated per glucose molecule. This yields an estimate of $(28/6) \times 10^{-17} = 47 \times 10^{-18}\text{ mol-CO}_2\text{ sec}^{-1}$ in completely aerobic conditions. Assuming the volume of the IS to be 1.6 pL, CO_2 production would be about $30\text{ }\mu\text{M sec}^{-1}$. At low enough pH, CO_2 would of course largely diffuse out without being hydrated, but it should be noted that carbonic acid has a pK value as low as 6.1. Diffusion also much depends on the milieu: CO_2 escapes easily from a well-perfused cell, but in the poorly vascularized retinal tissue, the concentration is liable to build up.

(2) Anaerobic glycolysis yields 2 ATP and 2 lactic acid molecules per glucose molecule. Thus lactic acid is produced at the turnover rate of ATP, $28 \times 10^{-17}\text{ mol sec}^{-1}$, or $170\text{ }\mu\text{M sec}^{-1}$. As some of the lactic acid may escape directly through the membrane, this is an upper-limit estimate for the H^+ load from glycolysis.

In a steady state, resynthesis of GTP from GMP will take up hydrogen ions at the same rate as they are generated in the OS by the sequence of reactions from GTP to GMP. However, the enzymes converting GMP to GDP (guanylate kinase) and GDP to GTP (nucleoside diphosphokinase) are mainly located in the IS (Berger, DeVries, Carter, Schulz, Passonneau, Lowry & Ferrendelli, 1980). Thus, this reutilization of hydrogen ions cannot relieve the acid load of the OS. On the other hand, of course, part of the acid produced in the IS will be consumed by GTP synthesis.

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