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LIGHT-TRIGGERED PROTON MOVEMENTS IN RETINAL DISCS FROM THE FROG

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SUMMARY

Illumination of retinal discs from dark adapted frogs caused a rapid and transient alkalinization of the suspension medium, followed by a slower and more prolonged acidification. Pretreatment with valinomycin, gramicidin or a proton conductor did not change this pattern, but the initial proton uptake was prevented by concentrations of a detergent or alcohol that had no effect on the acidification process. The initial transient proton uptake corresponded to a minimum of 5.7 H^+ taken up per rhodopsin bleached, with an elevation of this stoichiometry at early times of illumination. It is suggested that bleaching of rhodopsin contained in retinal discs initiates a transmembrane ion flux that is reflected by a net proton entry.

INTRODUCTION

The major complement of rhodopsin in the vertebrate rod outer segment is contained as an integral membrane protein in the intracellular retinal discs (1). Because the primary photopigment is separated physically from the plasma membrane, where eventual changes of ionic permeability must occur after illumination, it is thought that rhodopsin bleaching alters cellular levels of a substance that migrates from the discs to the plasma membrane (2-4). One set of observations implicates a series of scalar reactions. In this case, attention has centered on possible role(s) of guanine nucleotides, since microinjection of cGMP can depolarize the cell and extend the latency of later hyperpolarization by light (5). Moreover, retinal discs contain a phosphodiesterase that is markedly stimulated by light (6,7), and intact outer segments display a vigorous light-activated GTPase activity (8). Other findings suggest that a vectorial process might occur in which cytoplasmic Ca^{2+} is elevated by its release from within the discs. Both indirect and direct tests show that increased Ca^{2+} activity can regulate ion permeability at the

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plasma membrane (2,9,10), sufficient Ca^{2+} is associated with or contained in the discs (11,12), and although demonstrations of light-modulated Ca^{2+} release have not been uniformly successful (11,13-16), studies with purified rhodopsin indicate the appearance of "channels" capable of accepting multivalent cations (17,18). In distinguishing between these alternatives it is important to decide whether vectorial or scalar reactions occur after bleaching of rhodopsin. Since it seemed likely that changes of external pH could reflect the release of a stored "transmitter" such as Ca^{2+}, the experiments reported here have examined possible proton movements associated with illumination of freshly isolated discs.

MATERIALS AND METHODS

Bullfrogs (*Rana catesbeiana*) were dark adapted overnight. On the morning of an experiment, retinas from 2-5 frogs were excised under dim red light and gently shaken in an unbuffered amphibian Ringer (112 mM NaCl-3.4 mM KCl-2.7 mM $CaCl_2$). After allowing debris to settle (10 min), the supernatant containing rod outer segments was centrifuged at 20,000g for 10 min. The retinal discs were liberated following resuspension of the pellet in 5 mM $MgCl_2$, along with a brief homogenization, before addition of NaCl (or KCl) to 120 mM. It is presumed that retinal discs are released by osmotic lysis of resealed outer segments, but that the discs themselves do not lyse (11). Protein content of the final preparation was 100-300 µgm/ml; pH was about 6.5. Although experiments were performed at room temperature (23°C), discs were kept at ice bath temperature during preparation and storage. The work was done as soon as possible after sacrifice of the animals (1-2 hr), since apparent proton uptake after illumination tended to decay with continued storage. For the initial work (e.g., Figs. 1 & 2) 2 ml of a disc suspension was placed in a small glass vial along with a magnetic flea, and the vial fixed to the surface of a wooden block laid on a magnetic stirrer. If necessary, pH was adjusted to about 6.5 at this point. Medium pH was measured with a combined glass electrode (Radiometer GK2321C), using a pH meter (Radiometer PHM 63 or 64) whose output was displayed on a Linear Instruments recorder at 0.1-0.2 pH units full scale. After temperature equilibration, room light (28 footcandles: 440 erg/cm^2sec) was used for illumination, and at the end of the experiment the pH tracings were calibrated by additions of standardized HCl. The same general procedures were followed in cases where rhodopsin content was also determined, except that illumination was provided by a beam of unfocused white light at incident light intensities of 2-20 footcandles. To determine unbleached rhodopsin content, aliquots were taken before and after illumination, centrifuged as above, and the pellets solubilized in 10% digitonin. Absorbance at 500 nm was then measured before and after complete bleaching, and the change in absorbance used to calculate unbleached rhodopsin levels using an extinction coefficient of 40,600. After full bleaching, residual absorbance at 500 nm was 10%-15% of the value prior to bleaching.

RESULTS

A complex response was observed when the pH of a suspension of retinal discs was measured during illumination (Fig. 1). An initial alkalinization was soon

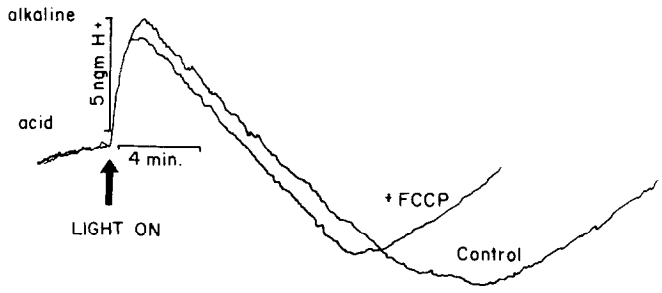

Figure 1. Changes of medium pH during illumination of suspensions of retinal discs. Discs were prepared as described in Materials and Methods. Fifteen minutes before illumination at 28 footcandles (room light) suspensions received either 2 μM FCCP (in ethanol) or the equivalent amount of ethanol (to 0.1%). The pH tracings are uncorrected for the small difference in buffering power of the two samples (280 ngm H^+/pH for the control; 340 ngm H^+/pH for the FCCP treated material). The pH of the samples was 6.4 - 6.5.

followed (30-60 sec) by a more prolonged acidification; this, in turn, was then followed by a return to the alkaline drift found before illumination. It appears significant (see below) that the initial transient alkalinization was complete at a time when visual inspection indicated considerable unbleached rhodopsin still present. Discs previously treated with 2 μM FCCP[+], a proton conductor, showed the same pattern (Fig. 1), and the other work (not shown) indicated comparable response after pretreatment with valinomycin (1-5 μM), gramicidin (1-5 μM) or K(or Na)SCN (30 mM). Such findings show that these light-triggered changes of pH do not arise from an active inward (or outward) pumping of H^+ alone.

The complexity of this response to illumination suggested that the pH changes result from more than one process initiated by illumination. In an attempt to distinguish possible vectorial and scalar reactions, the experiment shown in Fig. 1 was repeated in the presence of agents that might disrupt the permeability barriers present. Data from one such study are given in Fig. 2, where net changes are presented with reference to preillumination baselines. These results show that treatment with either alcohol (5% *n*-butanol) or a detergent (1% Triton X-100) abolishes the initial alkaline shift, but not the acidification phase. The same conclusion was reached in 4 other experiments where the detergent concentration was varied between 0.05% and 0.5%. In each case, illumination was abruptly

[+]FCCP, carbonylcyanidide-*p*-trifloromethoxyphenylhydrazone.

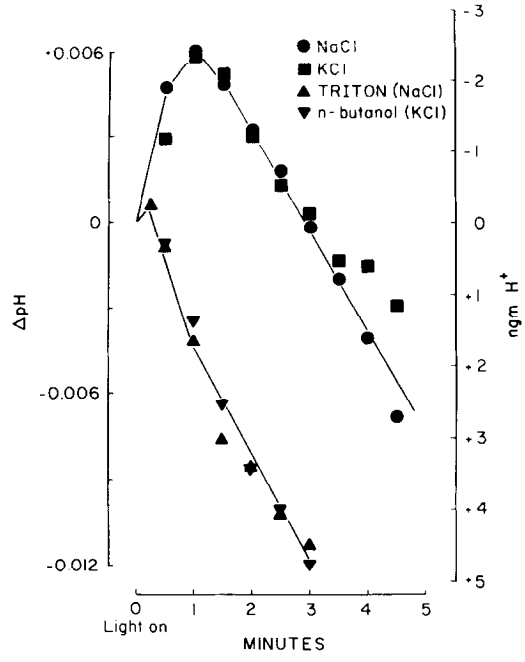

Figure 2. Effect of disruption of membrane permeability barriers on light-triggered pH changes. Suspensions were prepared as described in the legend to Fig. 1, using either 120 mM NaCl or KCl for final resuspension. Where shown, the suspensions were made 1% with Triton X-100 or 5% with *n*-butanol. Net changes in pH (left ordinate) or proton content (right ordinate) are given relative to extrapolated preillumination baselines.

followed by acidification, with little or no evidence of the alkalinization found in untreated controls. The results presented in Fig. 2 also show that neither the alkalinization nor acidification required Na^+ or K^+ as the predominant cation

The simplest explanation of these results would suppose that illumination initiates two distinct kinds of reactions: (i) a transient vectorial process, reflected by net proton uptake; and (ii) a more persistent scalar reaction(s) generating net acid. However, ascribing the transient alkaline shift to a vectorial process is unwarranted on the basis of results presented thus far. For example, in studies of frozen and thawed cattle discs, McConnell *et al.* (19) show that in the presence of 2% Triton, bleaching of rhodopsin is associated with either proton uptake or release, depending on pH (apparent null point of 6.4). In addition, these and other studies (20) confirm earlier reports (21,22) that near pH 7 in the absence of detergent there is a stoichiometric proton uptake of about 1 H^+/rhodophin bleached. Thus, it was important in the next experiments to

measure the stoichiometry of H^+ taken up as bleaching occurred, and to determine whether this value was fixed or if it depended on prior illumination.

In two additional experiments the kinetics of rhodopsin bleaching were determined after illumination for 30-120 sec at two different light intensities (5 and 20 footcandles). When normalized for constant light intensity (20 footcandles), the data indicated that unbleached rhodopsin disappeared with a half time of 100-120 sec. In these same experiments, measurements of pH changes were made during illuminations of 15 or 30 sec. From the net alkalinization observed (compared only with preillumination baselines) and from the measured kinetics of bleaching, mean values of 6.8 and 3.9 H^+ taken up/rhodopsin bleached were calculated for the lower and higher light intensities, respectively (Fig. 3). In a third study, only initial rhodopsin content was measured, and it is assumed that the kinetics of bleaching in this case were comparable to those found earlier. In this last instance, net alkalinization was followed during the first 15 sec of

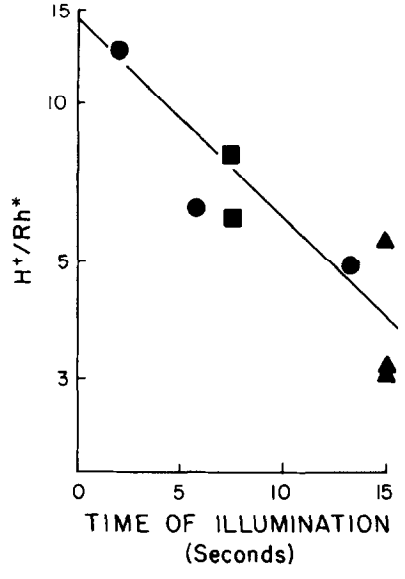

Figure 3. Stoichiometry of apparent proton uptake per molecule rhodopsin bleached. Data from three experiments are presented. In each experiment, net proton uptake (compared to preillumination baselines) was measured during an illumination at 2-20 footcandles for 15 or 30 sec. Individual experiments are shown with different symbols. In two cases (■,▲) separate samples were examined. In the third case (●) successive illuminations were performed using the same sample. The stoichiometry of H^+ taken up/rhodopsin bleached (H^+/Rh^*) is expressed relative to the equilivalent time of illumination at 20 footcandles.

successive illuminations at increasing light intensities. The initial trial, equivalent to 1.5 sec at 20 footcandles (no prior bleaching), gave a stoichiometry of 12 H^+ taken up/rhodopsin bleached. However, the last trial (15 sec at 20 footcandles, after cumulative illumination equivalent to 120 sec at 20 footcandles) gave 0 H^+ taken up/rhodopsin bleached. The mean value of all positive trials in this experiment was 6.3 H^+/rhodopsin. Taken together, data from these three separate experiments indicate 5.7 H^+ taken up/rhodopsin bleached, but two factors make it likely that this represents an underestimate. Such calculations do not consider the possibility that the alkalinization is accompanied by a simultaneous acidification (e.g., Fig. 2), and higher ratios are found at lower light levels.

DISCUSSION

The experiments summarized here suggest that a vectorial reaction occurs after bleaching of rhodopsin in freshly isolated retinal discs, and that this is reflected by a net proton entry. Three observations support this interpretation: (1) low concentrations of a detergent block apparent H^+ uptake; (2) H^+ uptake is limited in extent and complete before much of the rhodopsin is bleached; (3) the ratio of H^+ taken up/rhodopsin bleached is not fixed, but varies with illumination time and intensity. Tests with ionophores rule out the possibility that this H^+ uptake is an active electrogenic pumping, or that it arises from the coupled exchange of H^+ with monovalent cations such as Na^+ or K^+. These tests do not exclude coupled exchange between H^+ and divalent cations, coupled H^+ and anion entry, or passive H^+ movements as a secondary response to cation efflux or anion entry. This last possibility seems the most likely, since bleaching of both bacteriorhodopsin (23) and vertebrate rhodopsin (24) exposes a pathway for H^+ diffusion. It should be noted that if efflux of Ca^{2+} were to occur physiologically, then its replacement with discs by H^+ seems reasonable. If Ca^{2+} efflux were accompanied by overall cation exchange, undesirable osmotic effects might result if the entering cation were not buffered (bound) as well as the original Ca^{2+}. Of the monovalent cations available for Ca^{2+} exchange, only H^+ is likely to be bound as effectively as Ca^{2+} itself.

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