Biochimica et Biophysica Acta, 436 (1976) 140–153

BBA 77317

EFFECTS OF INCREASED INTRACELLULAR pH-BUFFERING CAPACITY ON THE LIGHT RESPONSE OF *LIMULUS* VENTRAL PHOTORECEPTOR

JONATHAN A. COLES* and JOEL E. BROWN

Department of Anatomy, Vanderbilt University, Nashville, Tenn. 37232 and Marine Biological Laboratory, Woods Hole, Mass. 02543 (U.S.A.)

(Received September 26th, 1975)

SUMMARY

Aspects of a possible involvement of hydrogen ions in the electrophysiological responses to light of *Limulus* ventral photoreceptors were investigated. A 1 M solution of either a zwitter-ionic pH buffer or a weakly-buffering control substance was pressure injected through a micropipette into a ventral photoreceptor cell. To estimate the amount injected, $^{35}SO_4$ was included in the solution. Membrane currents induced by light flashes were measured by a voltage-clamp technique. The buffer-filled micropipette passed current and a 3M KCl filled micropipette monitored membrane voltage. The sensitivity (peak light-induced current/stimulus energy) was measured, after dark adaptation, before and after injection. Injections of buffers, pH 6.3–7.2, to intracellular concentrations of at least 40–200 mM produced only a small mean decrease in sensitivity, approximately equal to that caused by injections of control substances. Excitation, therefore, apparently is not mediated by a change in intracellular pH. Buffers with pH values 5.4–8.4 were also injected. The time to peak of the response depended on pH, being shortened by up to 20 % at pH values below 7.7 and lengthened at higher pH values. The time to peak of the response appeared to be shortened by an increase in intracellular pH-buffering capacity even when there was no change in intracellular pH.

INTRODUCTION

The electrical potential in the soma of a photoreceptor cell of *Limulus* ventral eye can be held constant by means of two intracellular microelectrodes in a voltage-clamp circuit [1, 2]. In response to illumination there is a light-induced current through the surface membrane carried mainly by an influx of Na^+ [1, 3, 4]. At low

Abbreviations: EPPS, *N*-(2-hydroxyethyl)piperazinepropanesulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino) propanesulfonic acid; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid).

* Present address: Département de physiologie de l'université, Ecole de Médecine, 1211 Genève 4.

illuminances the light-induced current appears as discrete waves [3, 5]. The simplest hypothesis that explains the stochastic properties of discrete waves is that each one results from photoisomerization of a single rhodopsin molecule [6]. A discrete wave may have an amplitude of as much as 5 nA; it is unlikely that a single pore in the surface membrane could carry this current and still show the observed ion selectivity. At the frog neuromuscular junction each ion channel carries approximately 0.01 nA [7]. If the light-induced current in a *Limulus* photoreceptor flows through similar channels then on the order of 100 must open after photoisomerization of one rhodopsin molecule. A similar estimate has been obtained by assuming that the conductance of the ion channels in *Limulus* is similar to that of the Na^+ channels in squid axon [8, 9]. One possibility is that the excitation spreads from the rhodopsin molecule to the channels by means of a diffusible internal transmitter substance [8]. A similar hypothesis appears to be tenable for vertebrate rods and cones [10, 11, 12]. In the vertebrate (where Na^+ channels are closed as a result of illumination) there is indirect evidence that the internal transmitter is Ca^{2+} [11, 13], but in *Limulus* ventral photoreceptors an increase in intracellular free Ca^{2+} seems to play a different role, that of mediating the decrease in sensitivity caused by prior illumination (i.e., light adaptation) [14–16].

The light-initiated reactions of a number of systems containing rhodopsin-like pigments are associated with the movement of protons. For example, illumination of solutions of vertebrate rhodopsin [17–19], suspensions of rod outer segements [20, 21], or whole vertebrate retina [22] causes changes in the pH of the medium. In *Halobacterium halobium* absorption of light by bacteriorhodopsin results in movement of protons through the cell membranes [23, 24]. The first part of the present report is concerned with whether a local change in intracellular H^+ concentration is necessary for the generation of the electrophysiological response to light in *Limulus* ventral photoreceptors. If not, hydrogen ion may be ruled out as the hypothetical internal transmitter. We have tested this by increasing the intracellular pH-buffering capacity to a high level, so as to reduce any changes in intracellular pH that might normally occur during the response to light, and observing whether this treatment affects the sensitivity to brief flashes of light. To measure the sensitivity we chose not to record the receptor potential. At low illuminances the receptor potential shows quantum fluctuations, and if the illuminance is increased slightly a regenerative spike occurs. Hence, many measurements would have to be made to detect a small change in sensitivity. A more convenient indication of changes in the excitation process is obtained if the soma is voltage-clamped and the light-induced current recorded. In a dark-adapted cell, the peak light-induced current increases steadily with illuminance for illuminances up to at least two orders of magnitude above threshold [5].

The second part of this report describes changes in the time course of the light-induced current caused by injecting pH buffer, and the dependence of these changes on the pH of the buffer.

A preliminary report of part of this work has been published [25].

METHODS

The methods were based on those described by Lisman and Brown [2, 15]. A length of desheathed ventral rudimentary eye [26] was bathed for 1 min in a solution

of Pronase (20 mg/ml, B grade, Calbiochem, Los Angeles, Calif.) and pinned to a layer of Sylgard 184 (Dow Corning Corp., Midland, Mich.) in the bottom of a Petri dish containing about 5 ml artificial sea water of composition: NaCl, 423 mM; KCl, 10 mM; $MgCl_2$, 22 mM; $MgSO_4$, 26 mM; $CaCl_2$, 10 mM; $NaHCO_3$, 2.15 mM; Tris · Cl buffer, 10 mM; pH 7.8. The dish was placed on the stage of a microscope and an apparently isolated cell was positioned in the field. One glass micropipette containing 3 M KCl and a second containing the injection solution were brought up to opposite ends of the cell and made to penetrate the surface membrane by setting the capacitative feedback circuits of the amplifiers momentarily into oscillation. The experiments were done on cells in which the two electrodes recorded indistinguishable receptor potentials with a maximum amplitude of at least 70 mV. In some cells, meeting these criteria required repositioning one or both electrodes. The cell was allowed to reach a steady state of dark adaptation indicated by a constant sensitivity to weak test flashes; the amplitudes of the discrete waves were usually 1–10 mV. The membrane potential was then clamped to the dark level and flashes (10–20 ms) were presented at 30-s intervals. The series of flashes began at an intensity that produced a barely detectable response; the intensity of each successive flash was increased by 0.6 log unit until the light-induced current appeared to be approaching saturation. For some cells the weaker stimuli were presented several times, so that statistical fluctuations could, to some extent, be averaged out. From these data a response vs intensity graph was constructed that served as a control for the subsequent experiment. The membrane voltage was unclamped and a solution was injected by applying compressed nitrogen to the back of the micropipette. The immediate effects of the injection were monitored by observing the amplitude of the receptor potentials elicited by repetitive test flashes of constant intensity. The injection was usually begun at a low pressure (approx. 3 lb/inch^2, $10^{-4} \cdot m^{-1} \cdot kg^{-2}$) which was increased as necessary in steps up to a maximum of 30 lb/inch^2 ($10^{-3} \cdot m^{-1} \cdot kg^{-2}$) until the amplitude of the responses fell or the dark potential shifted. The time required for injection was usually about 1 min but ranged from a few seconds to more than 10 min. After an interval of 5 min for dark adaptation, the response vs intensity relation was again determined, the membrane potential being clamped to the same voltage as for the control.

Injection solutions As far as possible pH buffers were selected that (1) had pK values within 0.6 unit of the desired pH of the injection solution, (2) were not known to affect cell metabolism, (3) had a negligible affinity for Ca^{2+}, and (4) whose chemical structures were such that they were unlikely to pass through the cell membrane. Zwitterionic amino acids of the type introduced by Good et al. [27, 28] can meet these requirements. Buffer solutions, usually 1 M, were made with deionized water and with potassium as the cation; the pH values were measured with 100 mM buffer in 0.5 M KCl. HEPES, MES and MOPS were obtained from Sigma Chemical Co., St. Louis, Mo.; EPPS and PIPES from Poly-Sciences, Inc., Warrington, Pa.

Estimation of intracellular concentrations Carrier-free $H_2{}^{35}SO_4$ (New England Nuclear, Boston, Mass.) was included in the injection solution. After an injection the maximum concentration of $^{35}SO_4$ in the cell was in the order of 10^{-6} M. Immediately after the second determination of the response vs intensity relation the nerve was removed from the dish. It was placed in a vial and partially digested by Pronase. Scintillation fluid (Aquasol, New England Nuclear, Boston, Mass.) was added so

that the radioactivity could be measured. The volume of a large ventral photoreceptor cell body is about $4 \cdot 10^{-10}$ l [29]. We have estimated a lower limit for concentrations of injected solutions by assuming a volume of $5 \cdot 10^{-10}$ l.

RESULTS

The relation between amplitude of the light-induced current and stimulus intensity

The magnitude of the peak light-induced current was plotted as a function of light intensity, both scales being logarithmic. Fig. 1 shows typical examples. At the lowest intensities the response consists of only a small number of discrete waves, so there is considerable statistical fluctuation in the amplitude. At intensities about two orders of magnitude higher, the response shows signs of saturation. Usually the intensity run was stopped at this point in order to prevent long lasting light adaptation of the cell. In some of the cells that were stimulated at higher intensities a second limb of the curve appeared (similar to Fig. 2 of ref. 16).

Intensity runs were made before and after injecting buffer or control solutions into more than 100 cells. In some cases the resting potential shifted. There was no apparent relation between the sign and magnitude of the shift (after the 5 min dark adaptation) and the substance or quantity injected. In some further cells an electrode

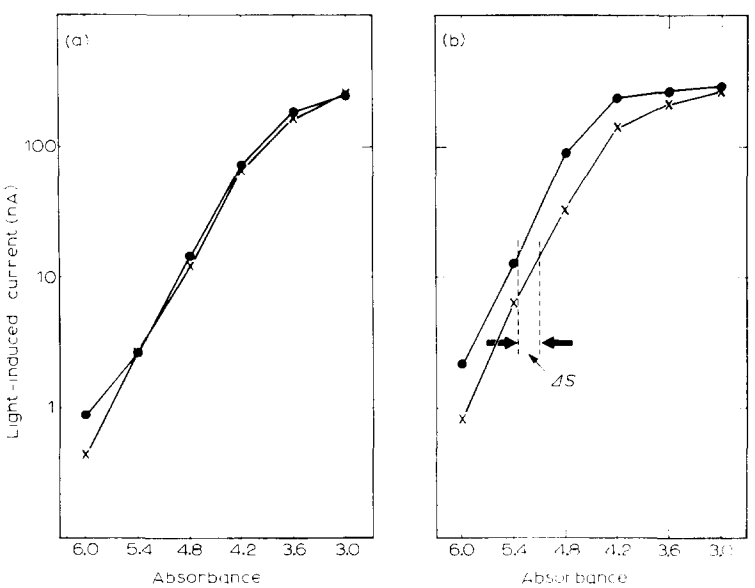


Fig. 1. Typical response vs intensity curves. (a) The ordinate is the peak light-induced current on a logarithmic scale. The abscissa is a logarithmic measure of the stimulus intensity: each number is the absorbance of the neutral density filter used to attenuate the light. ●, before injection. ×, after injection of PIPES buffer, pH 6.9, to an estimated intracellular concentration of 106 mM. (b) Curves obtained as for (a) from another cell. After injection of MOPS, pH 7.2, to an estimated concentration of 65 mM, there was a change in sensitivity. The approximate lateral displacement of the curves was measured at a value of the light-induced current about 1/20 of the estimated saturating light-induced current, as shown by the arrows. The light source was a quartz iodide lamp giving an unattenuated illuminance on the nerve of about $7.8 \cdot 10^3$ W/m^2; the stimulus duration was 20 ms.

came out of the cell during the injection: we suggest that injection could mechanically disturb the cell and that sometimes this caused a change in resting potential. The seven cells in which the potential shifted by more than 15 mV were excluded from the analysis.

Changes in the sensitivity to light caused by injecting pH buffers with pH values in the range 6.3–7.2

Fig. 1a shows the response vs intensity relations of a cell before and after injection of a buffer (PIPES) to an estimated intracellular concentration of at least 106 mM. In this cell there was no change in sensitivity. However, for most cells the two curves did not coincide. The change in the response vs intensity relation could always be described, approximately, as a displacement along the abscissa, as in the example in Fig. 1b. This displacement was measured for every cell at a value of the light-induced current about 1/20 of the estimated saturating light-induced current: for the cell of Fig. 1b it corresponds to a change in sensitivity of about −0.3 log unit.

Since injection of a volume of solution equal to a large fraction of the volume of the cell was expected to disturb the electrophysiology of the cell, it was necessary to determine the effect of injecting weakly buffering control solutions. For this purpose, we chose anions relatively impermeant to the surface membrane; these are

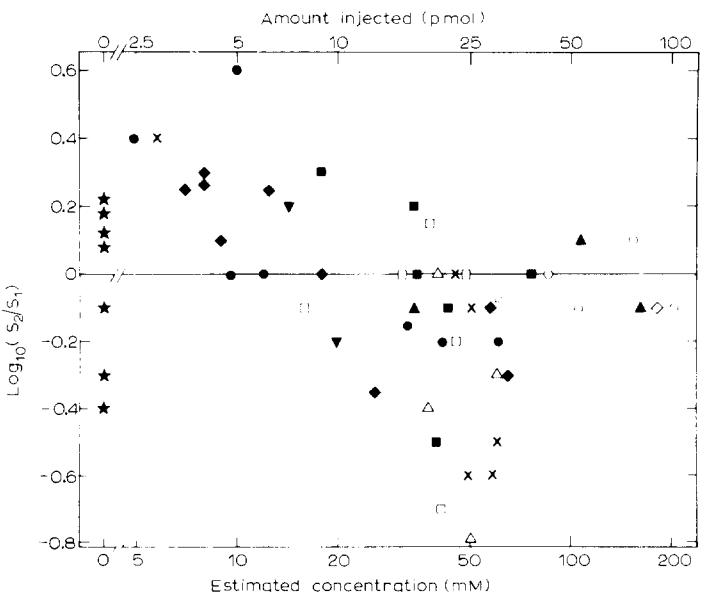
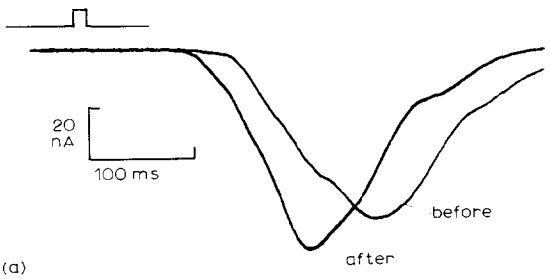


Fig. 2. Change in sensitivity vs amount of substance injected in the pH range 6.3–7.2. The top horizontal scale gives the amount of substance injected as calculated from the radioactivity in the nerve. The bottom scale gives the intracellular concentration, assuming a cell volume of $5 \cdot 10^{-10}$ l. The ordinate is the relative change in sensitivity after the injection, measured as illustrated in Fig. 1b. Each point was obtained from a different cell. Symbols correspond to solutions injected, as follows: ●, MES, pH 6.3, 1 M (pK 6.15); ■, PIPES, pH 6.3, 2 M (pK 6.80); ▲, PIPES, pH 6.94, 1 M; ▼, MOPS, pH 7.13, 2 M (pK 7.20); ◆, MOPS, pH 7.20, 1M ; ×, HEPES, pH 7.16, 1 M (pK 7.55); (Strongly buffering solutions). ○, K isethionate, 1.26 M; □, Li_2SO_4, 1 M; △, K_2SO_4, 0.6 M; ◇, Mannitol, 1 M; ★, no injection; (Weakly buffering solutions).

listed in the legend to Fig. 2. Values for the changes in sensitivity produced by injecting buffer solutions or control solutions are plotted in Fig. 2 as a function of the amount of substance injected.

Comparison of the effects of pH buffers and control solutions on the sensitivity

Thomas [30] found the intracellular pH-buffering capacity of *Helix* neurones, in the absence of extracellular HCO_3^-, to be equivalent to about 21 mM of a buffer



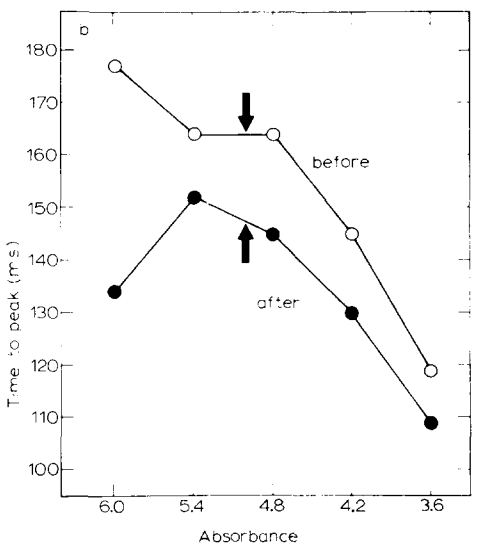


Fig. 3 (a) Time courses of responses before and after an injection. The figure shows superimposed photographs of oscilloscope traces of two light-induced currents from the same cell and for the same stimulus energy. The time of the stimulus is indicated by the deflexion of the upper trace. Each response was recorded in the course of determining a response vs intensity relation, as for Fig. 1. MES, pH 6.0, was injected to an estimated intracellular concentration of 80 mM. The stimulating light was attenuated by an absorbance of 4.8. (b) Time to peak as a function of stimulus intensity for a typical cell. The abscissa shows the absorbance of the filters used to attenuate the light. The ordinate is the time from the end of the 20 ms stimulus to the peak of the light-induced current. ○, before and ●, after injection of PIPES, pH 6.9, to an estimated intracellular concentration of 163 mM. The change in time to peak was measured, as indicated by the arrows, at the light intensity that produced 1/20 of the estimated saturating light-induced current before the injection. The points at the lowest intensities represent light-induced currents corresponding to only a few discrete waves and there was considerable variability in the latency.

at its pK. Assuming that the intrinsic buffering capacity of *Limulus* ventral photoreceptor cells is the same, injection of buffer to a concentration of 40 mM should nearly treble the buffering capacity. Injection of any solution, buffer or control, to an estimated intracellular concentration of more than about 300 mM usually caused a marked fall in sensitivity (see also ref. 15). To assess the effects of greatly increased intracellular buffering capacity not confounded by the effects of large injections, data were used only from the cells in which the estimated concentration was in the range, 40–200 mM. The mean change in sensitivity, when buffer solutions were injected, was −0.23 log unit (S.E. = 0.06, n = 14). When control solutions were injected the mean change was −0.21 log unit (S.E. = 0.10, n = 10). The statistical probability that the decrease in sensitivity caused by buffer is more than 0.3 log unit greater than that caused by the control solutions is about 0.015. This estimate of the probability was obtained by a t test, considering only one tail of the distribution (see, e.g. ref. 31).

Change in the time course of the light-induced current

Fig. 3a shows superimposed records of photoreceptor responses to two stimuli of the same energy, presented before and after injection of a pH buffer solution. After the injection, the time course changed, and, in this cell, the amplitude of the light-induced current increased slightly. Visual inspection of several such pairs of records suggested that, when there was a change in time course after an injection of pH buffer or a control substance, it corresponded approximately to a change in time scale: latency, time to peak and width of the response changed in unison. In order to compare the effects of different injections, the time from the end of the brief stimulus to the peak of the light-induced current was measured on each record; we call this the time to peak. As is typical of sensory receptors (see, e.g. ref. 32), the time to peak decreases as the stimulus energy increases (Fig. 3b). The change in time to peak was measured for the illuminance that produced 1/20 the saturating light-induced current before the injection; such a measurement is indicated in Fig. 3b. The change was expressed as a fraction of the time to peak before the injection.

The dependence of the changes in time to peak on the amount of substance injected

It would be of interest to know the relation between the change in time to peak and the intracellular concentration of injected substance. Because of the variability of the data it is necessary to consider many data points. Therefore points corresponding to injections of buffers with a range of pH values (6.3–7.2) have been plotted together on one graph (Fig. 4, filled circles). As will be shown below, the time to peak depends on the pH of the injected buffer, but the effect is negligible compared to the variability over this range of pH values. At estimated intracellular concentrations below 30 mM the injected buffers usually shortened, but for some cells lengthened the time to peak. In the range 30–200 mM the injection of these buffers consistently shortened the time to peak with no obvious dependence on concentration: the mean fractional change was −0.102 (S.E. = 0.019, n = 13). The effect of injecting control substances is shown by the open circles in Fig. 4. These tended to lengthen the time to peak. For concentrations in the same range (30–200 mM) the mean change was +0.091 (S.E. = 0.037, n = 11). It seems possible that not all the control substances produced the same effects: this is illustrated in Table I. For injections of buffer or control substance to less than 5 mM, the mean change in time to peak was −0.05

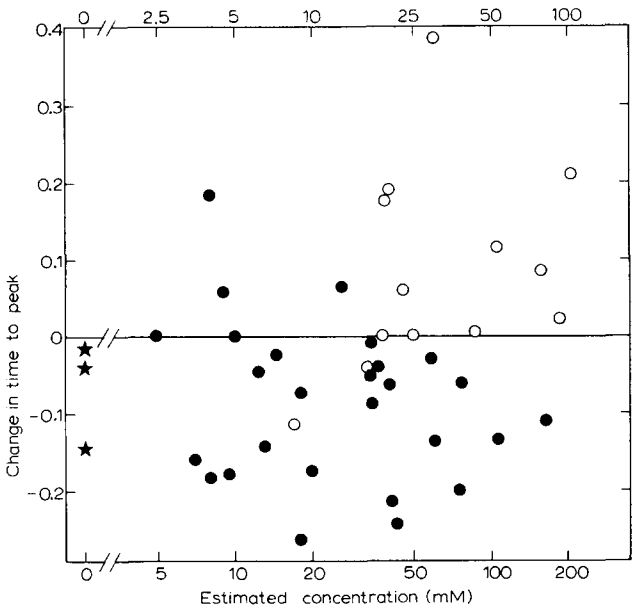


Fig. 4. Change in time to peak vs amount of substance injected in the pH range 6.3–7.2. The top horizontal scale gives the amount of substance injected and the bottom scale gives the estimated intracellular concentration, as in Fig. 2. The ordinate is the change in time to peak after the injection, measured as shown in Fig. 3b and expressed as a fraction of the time to peak before injection. Each filled circle represents a cell injected with buffer and each open circle a cell injected with control solution. The points are for 43 of the cells shown in Fig. 2. For the remaining 12, the times to peak were not measured because the responses were recorded only on a slow time scale or the sensitivity fell by more than 0.6 log unit.

TABLE I

EFFECTS OF CONTROL SOLUTIONS AT ESTIMATED INTRACELLULAR CONCENTRATIONS IN THE RANGE 30–200 mM

Cells in which log $S_2/S_1 < -0.6$ were excluded from calculation of the mean fractional change in time to peak.

Solution	Mean change in sensitivity: Log (S_2/S_1) (S.E. of mean)	Mean fractional change in time to peak (S.E. of mean)	Number of cells
Potassium isethionate	0.0 (0.06)	+0.068 (0.033)	3
Li_2SO_4	−0.13 (0.12)	0.005 (0.021)	5
K_2SO_4	−0.37 (0.16)	−0.25 (0.07)	4
Mannitol	−0.1	−0.024	1
Potassium citrate, pH 7.0	−0.18 (0.17)	−0.11 (0.12)	4*

* The citrate was not classed as 'weakly buffering'.

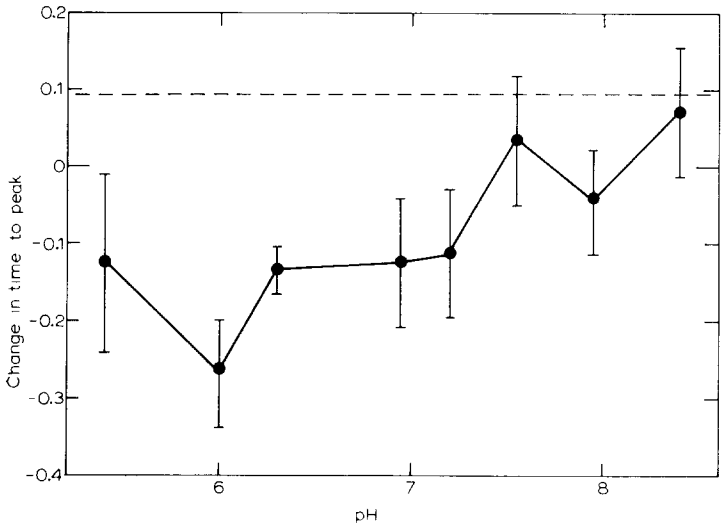


Fig. 5. Change in time to peak of the light-induced current as a function of the pH of the injected buffer. The ordinate is the change in time to peak, measured as shown in Fig. 3, and expressed as a fraction of the time to peak before injection. The abscissa is the pH of the buffer before injection. The buffers were injected to estimated intracellular concentrations in the range 40–200 mM. Each point represents the mean for several cells, except at pH 5.4, where there was only one cell. The error bars show standard errors, estimated as follows. When there were four or more cells at one pH (pH values 6.3 and 7.55) the S.E. was calculated in the usual way; then the mean S.D. for these pH values was calculated and taken to apply to the other pH values. In addition to the buffer solutions with pH values in the range 6.3–7.2, listed in the legend to Fig. 2, the following were used: citrate, pH 5.4 (1 cell); MES, pH 6.0 (3 cells); HEPES, pH 7.55 (4 cells); EPPS (pK 8.0), pH 7.95 (3 cells) and pH 8.4 (2 cells). The dashed line is the mean for the weakly-buffering control solutions.

($n = 4$). With very large injections, those causing an increase in intracellular concentration of more than 300 mM, time to peak was lengthened by both buffer and control substances. We presume that this, and the considerable loss in sensitivity usually observed with such large injections, may be due to a physical distortion of the cell [15]. In a few cells, the sensitivity changed by more than 0.6 log unit after an injection to a concentration less than 200 mM. We attribute most of this to unknown changes in the recording conditions and have excluded these cells from further analysis.

The effect of injecting pH buffers at pH values in the range 5.4–8.4

After injections to estimated intracellular concentrations above about 30 mM there was a tendency for the time to peak to be shorter at lower pH values and longer at higher pH values. This is illustrated in Fig. 5. The coefficient of the linear regression of fractional change in time to peak on pH was found to differ from zero at a level of of significance of 0.015.

DISCUSSION

The concentration of injected substance in the photoreceptor cell body

To determine whether the injected buffers contributed substantially to the

intracellular buffering capacity, we must estimate their concentrations in the cell bodies. We have assumed that the injected substance did not leave the cell body and that the radioactive marker, $^{35}SO_4^{2-}$, did not leave the nerve. However, some of the injected substances could have diffused down the axon. Measurements of passive diffusion of a substance in an axon have been reported for K^+ in squid giant axon [33]; a coefficient of diffusion of $1.5 \cdot 10^{-5}$ cm$^2 \cdot$ s^{-1} was obtained. The substances we injected formed ions larger than K^+ and presumably they had smaller coefficients of diffusion. If the value 10^{-5} cm$^2 \cdot$ s^{-1} is assumed for a substance injected into a cell body of volume $4 \cdot 10^5$ μm^3 and having an axon of diameter 20 μm [29], an approximate calculation [34] suggests that not more than 40 % of the substance will diffuse into the axon in 10 min. Injected substances may also have left the cell by passing through the plasma membrane. Measurement of the radioactivity in the artificial sea water in the chamber showed that it often contained less than one-fifth of the $^{35}SO_4^{2-}$ in the nerve. Since there was probably some leakage from the microelectrode before a cell was impaled this sets an upper limit on leakage of SO_4^{2-}. All the other injected anions were larger than SO_4^{2-} and, therefore, probably no more permeant. We suggest that the degree of overestimation of the concentration of injected substance in the cell body because of efflux is less than the error (usually an underestimate) introduced by assuming a single value for the volumes of all the cells. For most of the cells the concentration is probably not overestimated by more than a factor of two. It seems likely that the concentrations were uniform throughout the cell (Muller, K. J. and Brown, J. E., unpublished) have found that a molecule as large as horseradish peroxidase, injected intracellularly through a micropipette into a *Limulus* ventral photoreceptor, diffuses into the microvilli within 15 min.

Direct experimental demonstration that the pH buffers modified the intracellular environment of mechanisms involved in the generation of the light-induced current is given by the finding (to be discussed below) that the pH buffers affected the time course of the response. The further finding that this effect depended in a regular way on the pH of the buffer in the injection solution is evidence that, after injection, the buffers were actually buffering the intracellular medium.

The effect on the sensitivity of increasing intracellular pH-buffering capacity.

If the intrinsic pH-buffering capacity in *Limulus* ventral photoreceptors were much greater than that in other cells it would be inherently unlikely that changes in the intracellular pH were necessary for the functioning of the cell. Our assumption that the buffering capacity is equal to that in another invertebrate neurone [30] is therefore conservative. Hence, the injections of buffers to estimated intracellular concentrations in the range 40–200 mM presumably caused the intracellular buffering capacities to increase by factors in the range from nearly 3 to > 10. But the sensitivity of the photoreceptors was not decreased significantly more than it was by injection of weakly-buffering substances. The statistical probability is greater than 98.5 % that the true mean effect of the injected buffers is to decrease the sensitivity by a factor of < 2. But changes in hydrogen ion concentration should be approximately inversely proportional to the buffering capacity (see ref. 35), i.e., they should have been reduced by a factor, on average, of considerably > 3. We conclude that the light-induced conductance change in the dark-adapted ventral photoreceptor is not mediated by a

change in intracellular pH, or, in other words, that protons do not constitute the hypothetical internal transmitter.

The present experiments do not address the question of whether a change in intracellular pH, caused by intense or prolonged illumination, might mediate a contribution to the light-induced current in light-adapted photoreceptors. Neither do the experiments show that changes in intracellular pH play no part in light adaptation. The changes in sensitivity and time scale caused by light adaptation may be accounted for, at least qualitatively, by light-induced changes in the concentration of "free" intracellular Ca^{2+} [14–16], but a contribution from other factors is not ruled out.

Changes in time to peak of the light-induced current.

The injected solutions with pH values less than 7.7 may be divided into two classes: those that tended to lengthen the time to peak and those that shortened it. We have assumed that the relevant property of the substances that shortened the time to peak is that they are pH buffers. We can reject two different interpretations.

(*1*) *The intracellular calcium concentration was changed.* It is known that the effect of raising intracellular Ca^{2+} concentration is to reduce greatly the sensitivity of the cell and to decrease, by a relatively small proportion, the time to peak [36]. Since the buffers and the control solutions had similar, small effects on the sensitivity it is unlikely that one class caused a change in the intracellular Ca^{2+} concentration and the other did not.

(*2*) *The excitation process was affected by zwitterions.* At physiological pH values, all the buffers were partly in zwitterionic form but the control substances were not. To see if a non-zwitterionic buffer would have the same effect, we injected citrate at pH 7.0, where the divalent and trivalent ions predominate. Its buffering capacity at this pH should be only about 0.23 of that at its pK; also it is probably metabolized. Therefore the data for it were not included with those for the other buffers. Nevertheless, like them, it shortened the time to peak (see Table I). It appears, therefore, that the decrease in time to peak depended on the injection of pH buffer at a pH less than about 7.7.

The effect on the time to peak of changing intracellular pH

As shown in Fig. 5, the time to peak tended to shorten when a pH buffer at a pH lower than about 7.2 was injected, and tended to lengthen when a buffer at a pH higher than about 8.2 was injected. Except in the case of injection of a buffer at the (unknown) physiological pH, the pH at the time the responses were recorded after the injection was probably intermediate between the pH of the buffer solution and the normal physiological pH. Two factors would have contributed to this: (1) the intrinsic buffering capacity of the cell and (2) the tendency for the intracellular pH to return towards its normal value after having been altered by the injection (see ref. 37). Hence, the dependence of time to peak on intracellular pH is, if anything, probably more marked than the dependence on the pH of the buffer solution (shown in Fig. 5). We conclude that the time course of one or more stages in the excitation process leading from the photoisomerization of a rhodopsin molecule to the opening of conductance channels in the surface membrane is sensitive to intracellular pH. The binding of a proton to the site on metarhodopsin that controls its color cannot be one

of these stages because, as shown by Lisman et al. [38], in *Limulus* this site has access to the extracellular but not the intracellular medium.

The effect on the time to peak of increasing the intracellular buffering concentration

Despite the range of their chemical structures, the control substances all tended to lengthen the time to peak. It has been shown that there is no significant change in time to peak when a *Limulus* photoreceptor is made to swell by osmosis [39, 40]. But there may be some other consequence of the injection of any substance that tends to increase the time to peak. If so, then the same tendency should also be present after injection of buffer, and changes observed after injection of buffer should be compared with those caused by the control substances. As shown in Fig. 5, most of the pH-buffer solutions we used tended to shorten the time to peak and even those (at the higher pH values) that tended to lengthen the time to peak did so less than the control solutions. It might be expected that injection of a pH buffer at physiological pH would have the same effect as injection of a control substance. However, inspection of Fig. 5 suggests that this assumption would give as a lower limit for an estimate for the physiological pH a value of about 7.5. Reported values for intracellular pH in other animal cells are lower than this [41]. Our results therefore suggest that increased intracellular pH-buffering capacity in itself shortens the time to peak. The statistical significance that can be calculated for this conclusion depends on the value assumed for the normal intracellular pH. If this is 7.0 or less, then the statistical significance is high. To make a more conservative assessment of the data we can allow for the possibility that in our experiments the normal intracellular pH was as high as 7.6. The mean fractional change in time to peak was calculated for the injections of buffers with pH values in the region of 7.6 (7.2, 7.55 and 7.9 with a weighted mean of 7.6). The value was -0.025 ($n = 9$, S.E. $= 0.044$). The effect of the control solutions was a change of $+0.091$ (S.E. $= 0.037$, $n = 11$). The difference between these two values is significant at the 5 % level.

A possible mechanism for the effect of increased intracellular pH-buffering capacity

There is a class of chemical reactions whose rates tend to be increased when the pH-buffering capacity of the medium is increased. These are reactions at fixed sites at which there is uptake or release of protons, weak acids or weak bases: transport of these radicals through a solution is effectively facilitated by the presence of pH buffer (Engasser and Horvath, ref. 42). This concept of buffer-facilitated transport has been used to explain observed rates of transport into cells (e.g. ref. 43 and reaction velocities of enzymes bound to artificial membranes [44–46]). We suggest that it also provides a hypothesis that might explain our finding that the excitation process in *Limulus* ventral photo-receptors appears to be more rapid when the intracellular pH-buffering capacity is increased: the behaviour is, qualitatively, what might be expected if part of the excitation process was the uptake or release of protons, a weak acid or a weak base, at fixed, intracellular sites. Borsellino et al. [47] have shown how the waveform of the receptor potential of eccentric cells in *Limulus* lateral eye might be accounted for if each absorbed photon set in train a series of chemical reactions. Conceivably, there is an analogous series of reactions in ventral photoreceptors and changes in intracellular buffering capacity affect one or more steps.

The degree of the increase in reaction rate brought about by the addition of

buffer can be several fold and depends on the rate of the reaction (when not diffusion-limited) and the geometry of the surface on which it occurs. Silman [ref. 48, Fig. 5] shows a relative increase in reaction rate of membrane-bound acetyl cholinesterase of 2.6 after the addition of buffer. In his experiment, the reaction rate saturated when the buffer concentration was about 2 mM: a faster reaction occurring at geometrically less accessible sites (such as in a microvillus) might not have saturated completely at the physiological intracellular buffering capacity. In this hypothetical reaction a further increase in buffer concentration would produce a small increase in reaction rate, such as we have reported for the amplification mechanism of the light response.

CONCLUSIONS

We conclude that the light-induced opening of conductance channels in the surface membrane is probably not mediated by a change in intracellular hydrogen ion concentration. However, the kinetics of the excitation process appear to be affected by changes in intracellular pH, and probably also by increases in intracellular buffering capacity even when there is no change in intracellular pH.

ACKNOWLEDGEMENTS

We thank Drs. R. Hubbard, S. E. Ostroy and L. H. Pinto for helpful criticism of drafts of the paper and Drs. P. J. De Weer, J. E. Lisman, R. M. Miura and G. Strichartz for useful discussion. The work was supported by N. I. H. grants EY-00834 and EY-00835.

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