Chemical Excitation of *Limulus* Photoreceptors

II. Vanadate, GTP-γ-S, and Fluoride Prolong Excitation Evoked by Dim Flashes of Light

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ABSTRACT We treated *Limulus* ventral photoreceptors with the phosphatase inhibitors fluoride, vanadate, and GTP-γ-S [guanosine-5′0-(3-thiotriphosphate)] under various conditions of illumination and external calcium concentrations. In the dark in low-calcium (1 mM) artificial seawater (ASW), fluoride-induced discrete waves cluster together in time. Under these conditions, the intervals between waves were found to be correlated, and there were excess short intervals beyond the number expected from an exponential interval distribution. To assess the effects of the inhibitors on the light response, we stimulated ventral receptors with a series of dim flashes and averaged the current response under voltage clamp. In ASW, vanadate and GTP-γ-S prolong the decay of the averaged response to dim test flashes, but prolongation does not always accompany the induction of discrete waves in the dark. Prolongation induced by vanadate in normal-calcium (10 mM) ASW was enhanced in low-calcium (1 mM Ca^{2+}) ASW. Many individual response records suggest that prolongation results from extra discrete waves late in the light response, whereas others reveal long-lasting complex waveforms that cannot easily be resolved into discrete waves. The apparent effect of the inhibitors on the light response is to allow a single photoactivated rhodopsin molecule to produce multiple discrete waves and complex long-lasting events. We suggest that both prolongation of the light response and clustering of waves in the dark result from inhibition of a step in the pathway of visual transduction, in which GTP hydrolysis normally helps to turn off the production of both light-evoked and spontaneous waves.

INTRODUCTION

In the accompanying article (Corson and Fein, 1983), we have proposed a

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model in which certain phosphatase inhibitors elicit discrete waves in the dark by inhibiting a molecule that normally turns off the production of both spontaneous and light-evoked discrete waves in *Limulus* photoreceptors. Because the proposed site of action is directly in the pathway of phototransduction, the model predicts that the response to light should be modified by these compounds and especially by hydrolysis-resistant analogues of GTP. Our initial findings with fluoride indicated that the light response was prolonged, and that there was clustering among fluoride-evoked discrete waves in the dark (especially in low-calcium ASW). These two observations suggested to us that prolongation of the light response might occur by a fundamental alteration of transduction and this led us to the experiments reported here.

Previous analyses of discrete waves in invertebrates suggest that only a single photon is required to activate one rhodopsin molecule and that only a single discrete wave is produced by a photoactivated rhodopsin molecule under normal conditions (Yeandle, 1958; Fuortes and Yeandle, 1964; Scholes, 1965; Yeandle and Spiegler, 1973; Weiss and Yeandle, 1975; Lillywhite, 1977). It follows from the latter of these two hypotheses that the intervals between light-evoked discrete waves should have an exponential frequency distribution and should occur in uncorrelated sequences given the random and independent arrival of photons from a steady light source. Under normal conditions in *Limulus* photoreceptors, the intervals between light-evoked waves indeed appear to be exponentially distributed (Fuortes and Yeandle, 1964; Yeandle and Spiegler, 1973) and pair-wise independent (Yeandle and Spiegler, 1973). Because fluoride-evoked waves appear to cluster together in time under some conditions (Fein and Corson, 1979), we undertook an analysis of the intervals between discrete waves evoked by fluoride as well as the intervals between spontaneous and light-evoked waves in cells exposed to normal-calcium ASW and to low-calcium ASW.

Dim flashes of light ordinarily elicit discrete waves in a time-dependent Poisson manner, and the expected distribution of the numbers of evoked waves has been calculated (Weiss and Yeandle, 1975) and used in a direct test of the hypothesis that a single photoactivated rhodopsin produces, at most, one discrete wave under normal conditions (Lillywhite, 1977). Unfortunately, this approach breaks down in the presence of a high background rate of discrete waves such as that elicited by the phosphatase inhibitors. Nonetheless, information can still be extracted from individual records and averages of the responses to flashes of low intensity. We have taken this latter approach to examine the effects of phosphatase inhibitors on the light response.

A preliminary account of these results has appeared in abstract form (Corson and Fein, 1982).

METHODS

The methods for stimulating and recording from ventral photoreceptors have been reported previously (Fein and Charlton, 1975, 1977). They are similar to those

originally used by Millecchia and Mauro (1969*a*, *b*). The methods for injecting GTP-γ-S, for applying vanadate, and for counting discrete waves have been given in preceding papers (Fein and Corson, 1981; Corson and Fein, 1983).

Discrete waves evoked by light have an exponential interval distribution (Yeandle and Spiegler, 1973). The expected exponential probability density function, $g(t)$, for interwave time intervals governed by a time-independent Poisson process is given by Eq. 1:

$$g(t) = \lambda exp(-\lambda t), \tag{1}$$

where λ is the average rate of waves and t is the interval between the rising phases of successive waves. For the total number of waves, N, in an experiment of duration T, λ was estimated by N/T. The average rate, λ, was then used to calculate the expected number (EX) of time intervals within a bin, having a fixed increment of duration $(t_i - t_{i-1})$. The expected value was calculated by integrating Eq. 1 over the bin widths. In these experiments the bin width was chosen to be 40, 200, or 1,000 ms, in order to give a convenient number of bins, depending on the rate. Where EX values were <5, expected values of successive bins were summed until the expected value of the sum exceeded 5. This procedure generated n EX values, which are represented as dots on the interval histograms of Figs. 1 and 3. The experimentally observed intervals were then sorted into corresponding bins, which are given in the interval histograms. From the histograms of observed intervals, n observed values (OB) corresponding to the n expected values (EX) were tabulated.

The goodness of fit of our observed interval distributions to the expected exponential interval distribution was determined by chi-square analysis. The null hypothesis (that each interval distribution was truly exponential with a rate of $\lambda = N/T$) was tested using this statistic at the 0.05 probability level for $n - 2$ degreees of freedom. Under this procedure there was a <5% chance of rejecting the null hypothesis of a truly exponential distribution by mistake (type I error; Afifi and Azen, 1972, p. 320). Because we could not anticipate the forms of alternatives to the exponential distribution, we could not calculate the probability of accepting a nonexponential interval distribution as exponential by mistake (type II error). To minimize the probability of a type II error, we maximized the sample size for intervals (~500) within the expected life of the preparation.

The drift in the discrete-wave rate could give rise to a nonexponential interval distribution. Therefore, when we found a nonexponential interval distribution, we tested for a drift in the rate of occurrence of waves by dividing the record in half and comparing the rates in the two halves. We tested for equality by calculating a test statistic u given in Eq. 2, which is taken in slightly modified form from expression 6 of Cox and Lewis (1966, p. 228):

$$u = \frac{(n_1/t_1 - n_2/t_2)}{(n_1/t_1^2 + n_2/t_2^2)^{1/2}}. \tag{2}$$

In Eq. 2, n_1 and n_2 are the number of observed counts in intervals t_1 and t_2, respectively. For a large value of n, this test statistic is distributed approximately normally with a mean of 0 and a standard deviation of 1. The calculated value of u was compared with the critical value for u (1.96) from the normal distribution at the 0.05 probability level. Light-evoked increments in discrete-wave frequency above the background frequency can also be tested for equality by simple extension of Eq. 2 for linear combinations of rates.

We tested for correlations among the intervals by calculating the autocorrelation coefficient, r_1, for a lag of one interval between waves, and evaluating the significance of the correlation by means of a 95% confidence interval under the normal approximation, according to the procedure described by Cox and Lewis (1966, pp. 164–165).

The average flash response waveforms for each of the experimental conditions in this study were obtained in the following manner. After dark adaptation, 50 20-ms test flashes just bright enough to elicit a reliable response were given at 10-s intervals. The light-induced currents arising from cells voltage-clamped to their resting potentials were recorded on an FM tape recorder (model Store 4-DS; Racal, Rockville, MD). Response currents from cells voltage-clamped to their resting potentials were measured to ensure that we analyzed the effects of drugs only on the light-activated conductances and not on the voltage-activated ones. Responses were filtered by two stages of a single-pole low-pass filter with a 10-ms time constant. 2 s of the response beginning at the leading edge of the test flash were sampled at 2-ms intervals and averaged over the 50-flash sequence using a MACSYM computer (Analog Devices, Inc., Norwood, MA). Averaged waveforms were normalized and displayed on an oscilloscope screen. Following the usual convention, the inward current of the light response under voltage clamp is displayed as a downward deflection. Accordingly, the latency of the response is defined here as the time from the leading edge of the test flash to the time required for the average response to attain 5% of its peak amplitude. Similarly, the rise time is the time required for the response to traverse from 5 to 95% of its peak amplitude during the leading edge of the response. The decay time is then the time required for the response to traverse from 95 to 5% of its peak amplitude during the trailing edge of the response.

RESULTS

Interval Distributions of Discrete-Wave Occurrence

We have examined the interwave interval distributions of spontaneous, light-induced, and fluoride-induced discrete waves in normal-calcium ASW. As shown in Fig. 1, all three interval distributions appear to be exponential. Chi-square analysis (see Methods and Fig. 1) of the fit of the observed distributions to the expected exponential distribution calculated from the rates reveals no significant ($P > .05$) deviations from the expected exponential distribution. Thus, in normal-calcium ASW and at moderate fluoride concentrations, fluoride-induced discrete waves tend to have a simple exponential interval-distribution similar to that of spontaneous and light-induced discrete waves. We note, however, that fluoride-evoked waves sometimes cluster together in time in normal-calcium ASW, especially at concentrations of fluoride >5 mM.

We continued our analysis of the interval distributions in low-calcium ASW in the cell just described, as shown in Figs. 2 and 3. Frequent clusters of discrete waves, which we call bursts, appeared in the presence of fluoride (Fig. 2*C*), but not in its absence (Fig. 2, *A* and *B*). Examination of the interval distributions of spontaneous, light-induced, and fluoride-induced discrete waves reveals an excess of short intervals only in the presence of fluoride (Fig. 3*C*). Chi-square analysis of these interval distributions (Fig. 3) confirms

a significant deviation from the expected exponential only for the intervals between fluoride-induced waves.

Clusters of discrete waves were distributed throughout the record from

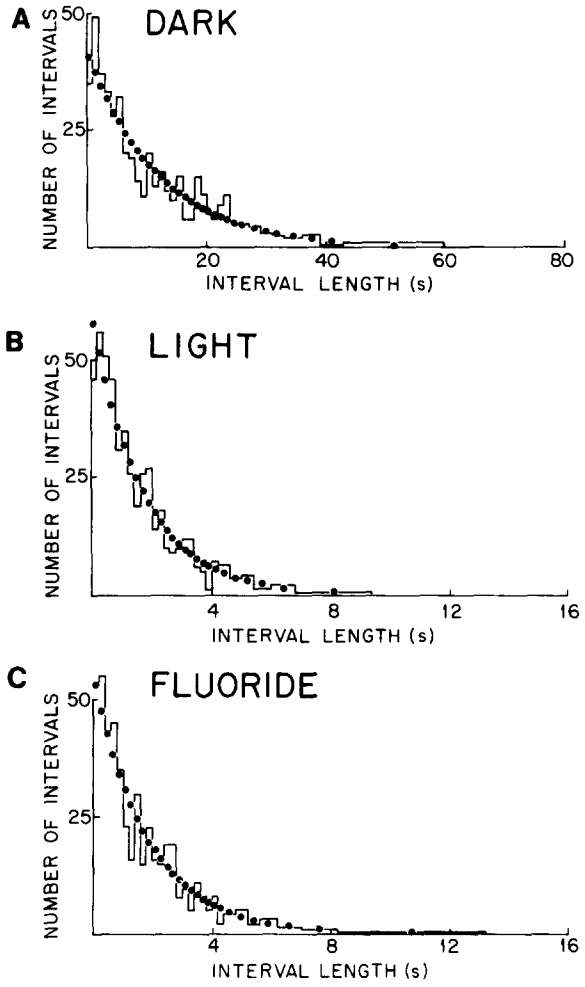


FIGURE 1. Distributions of time intervals between discrete waves that occurred spontaneously (*A*) or were induced by light (*B*) or 5 mM fluoride (*C*) in normal-calcium ASW. The dots represent the expected values calculated (see Methods) for an exponential distribution arising from a Poisson process with a rate $\lambda = N/T$. In these histograms, the values of N/T were 500/5,902 s, 515/863 s, and 511/935 s for *A*, *B*, and *C*, respectively. The calculated chi-square statistics (and degrees of freedom) used to test the goodness of fit to exponential hypotheses were 39 (31), 28 (25), and 28 (27) for parts *A*, *B*, and *C*, respectively. A fourth measurement (not illustrated) gave values of N/T of 546/500 s for the simultaneous exposure of the cell to light and fluoride. The calculated value of the *u*-statistic (see Methods) used to test for equality of the two light-induced increments was 0.56. Values of the autocorrelation coefficients for a lag of one interval were +0.06, +0.02, and −0.01 for conditions *A*, *B*, and *C*, respectively.

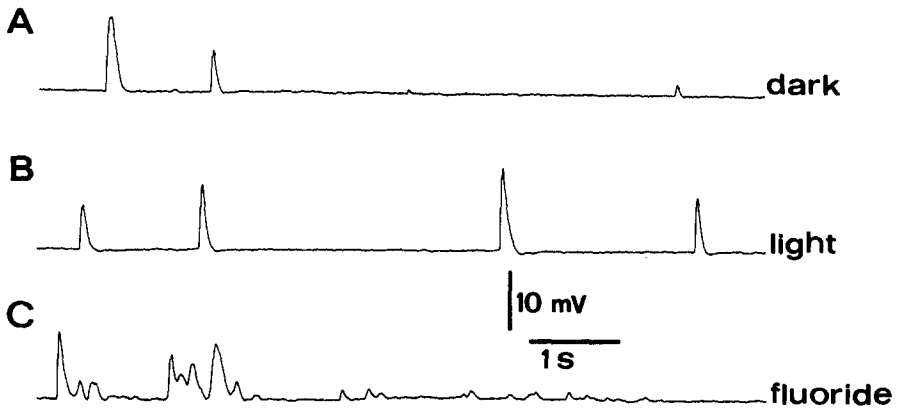


FIGURE 2. Clusters of discrete waves elicited by 5 mM fluoride in low-calcium (1 mM) ASW (*C*). Spontaneous (*A*) and light-induced (*B*) discrete waves recorded in low-calcium ASW after removal of fluoride do not exhibit clustering. Note that because record *C* was selected to illustrate bursts of waves, the rate of occurrence of waves in this interval appears to be higher than the overall rate for the entire experiment duration. The steady light stimulus in record *B* was attenuated 8.1 log units below the maximum available intensity. Records *B* and *C* were taken at a bath temperature of 20°C. Record *A* was made at 27°C (see legend of Fig. 3).

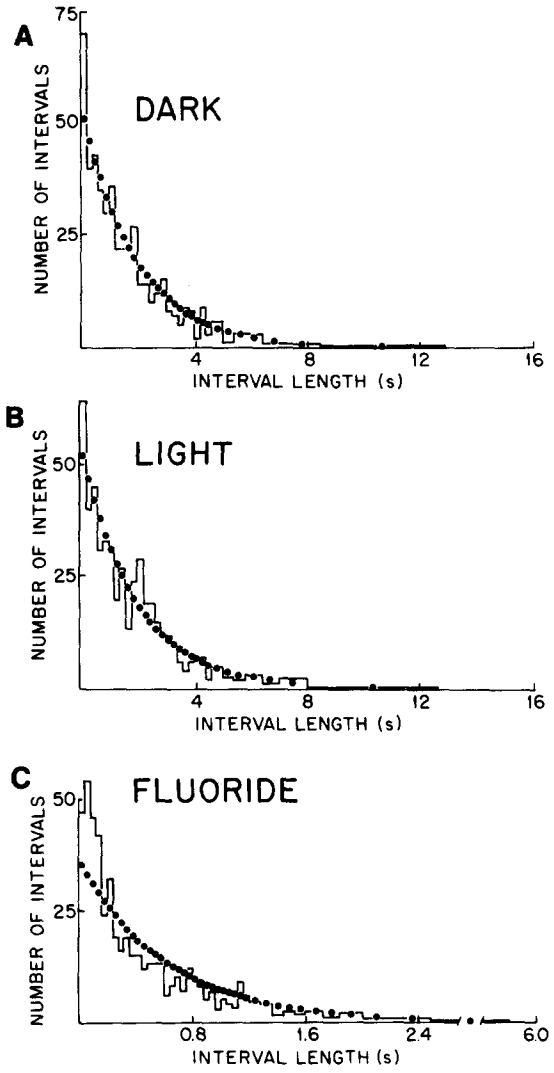
which Figs. 2*C* and 3*C* were derived. Nevertheless, we looked for a drift in the overall rate (see Methods and Fig. 3), because that might also be a source of excess short intervals. No evidence of drift was found. Approximately two-thirds of the observed waves would have to occur in one half of the record to generate excess short intervals comparable to these observed in the first four bins of Fig. 3*C*. From a calculation of the power of the test under the normal approximation (Afifi and Azen, 1972), there is little ($P \ll .05$) chance

FIGURE 3. (*opposite*) Distributions of time intervals between discrete waves that occurred spontaneously (*A*) or were induced by light (*B*) or fluoride (5.5 mM) (*C*) in low-calcium (1 mM) ASW. The dots represent the expected values calculated for an exponential distribution from a random process with a rate $\lambda = N/T$. There are excess short intervals among the discrete waves elicited by fluoride. In these histograms, the values of N/T were 514/979 s, 252/1,008 s, and 558/341 s for *A*, *B*, and *C*, respectively. The chi-square statistics (and degrees of freedom) used to test the goodness of fit to exponential hypotheses were 33 (28), 34 (28), and 69 (39) for parts *A*, *B*, and *C*, respectively. In part *C*, 257 waves occurred in the first half of the record and 301 occurred in the latter half. The calculated value of the *u*-statistic used to test for the equality of rates in the two halves of the record was 1.86. Parts *B* and *C* were done at a bath temperature of 20°C. Part *A* was done at 27°C because the spontaneous rate (0.04 discrete wave/s) at 20°C would have required an unreasonably long length of time (an additional 3.5 h) to accumulate 500 intervals. Values of the autocorrelation coefficients for a lag of one interval were −0.02, 0, and +0.171 for conditions *A*, *B*, and *C*, respectively.

of failing to detect a discrepancy of that magnitude. Therefore, it is unlikely that a drift in the rate contributes significantly to the excess of short intervals beyond that resulting from the clusters.

Events having a nonexponential interval distribution are not necessarily correlated. We tested for correlations among successive discrete waves using the autocorrelation coefficient for a lag of one interval (see Methods). By this test, the fluoride-evoked waves in low calcium (Fig. 3*C*) were found to be significantly correlated, and this correlation among waves constitutes a second kind of deviation from a simple Poisson process, in addition to the nonexponential interval distribution.

Conversely, events that have an exponential interval distribution are not



necessarily uncorrelated. We tested for correlations, and, under normal-calcium conditions, successive fluoride-evoked waves (Fig. 1*C*) were not significantly correlated, nor were spontaneous and light-evoked waves in both normal-calcium and low-calcium conditions (Figs. 1, *A* and *B*, and 3, *A* and *B*). The lack of correlation among successive waves under these conditions is consistent with a Poisson process in each case.

There was no evidence that appreciable changes in the sensitivity of the cell occurred during the course of the interval distribution measurements. The amplitudes of responses to 10 20-ms test flashes were averaged six times

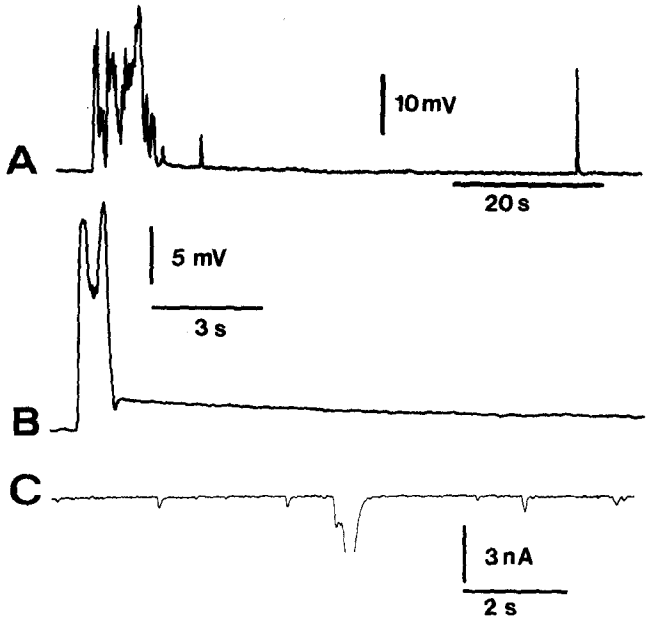


FIGURE 4. Bursts of discrete waves observed in *Limulus* photoreceptors. A particularly dramatic burst occurred spontaneously in a cell exposed to 400 μM vanadate in 1 mM Ca^{2+} ASW (*A*). One of the several naturally occurring discrete-wave bursts in an untreated cell in 10 mM calcium ASW is shown in *B*. In record *C*, an unusually large complex event (which went off the scale) occurred during the course of light-induced dark noise elicited from a ventral receptor (see Fein and Hanani, 1978).

during the course of the experiment (Figs. 1–3) and were found to be essentially constant.

We have observed bursts that are similar to and sometimes more dramatic than those of Fig. 2*C* in cells treated with vanadate and low calcium (Fig. 4*A*). GTP-γ-S was also capable of eliciting bursts of discrete waves in low-calcium ASW (results not shown). Bursts also appear to occur naturally in rare, untreated cells (Fig. 4*B*) and sometimes during light-induced dark noise (Fig. 4*C*) (Fein and Hanani, 1978). Bursts similar to that of Fig. 4*B* have also been observed by J. E. Lisman and M. A. Goldring (personal communication).

Bursts of discrete waves may be a natural but rare phenomenon that is accentuated when cells bathed in low-calcium ASW are exposed to fluoride, vanadate, or GTP-γ-S.

Linear Summation of Discrete-Wave Rates in ASW

From Figs. 1–3, it is clear that fluoride greatly increases the frequency of occurrence of discrete waves recorded in the dark. One question that immediately arises from this observation is whether fluoride will alter the number of discrete waves evoked by a steady light stimulus. We therefore exposed the cell shown in Fig. 1 to dim steady light during exposure to fluoride. We tested for equality of the light-induced increments (light minus dark) in the presence and absence of fluoride by the procedure described in Methods. No significant changes in the light-induced increment was found. From a calculation of the power of the test under the normal approximation, there is little chance ($P < .05$) of failing to detect a discrepancy in the rates larger than one-half of the light-induced increment found in ASW (0.51 discrete wave/s).

We attempted a similar measurement of the effect of fluoride on the light-induced increment of discrete-wave frequency in low-calcium ASW, but we found that the rate of waves evoked by fluoride in the dark was elevated after the exposure to light. We could not tell whether this was an effect of light on the fluoride-induced frequency or whether the elevation of the dark rate was simply the result of a drift in the rate of fluoride-evoked waves after long exposure. Because of this problem, we did not pursue experiments with steady light, but turned instead to the analysis of responses to dim flashes of light.

Prolongation by Vanadate in 10 mM Ca^{2+} ASW

As we had previously observed that fluoride prolonged the response to flashes of light (Fein and Corson, 1979), we next chose to examine the effect of vanadate. This drug can be applied extracellularly and usually has reversible effects when used at low concentrations (<5 mM). We have analyzed the waveform of averaged responses to dim flashes to document the occurrence of prolongation and to see if we could distinguish the cause of prolongation among the following alternative sources: (*a*) an increase in the number of photoactivated rhodopsin molecules, (*b*) an increase in the latency of discrete waves, (*c*) an increase in the time course of individual discrete waves, or (*d*) an increase in the number of waves produced by an individual photoactivated rhodopsin.

As shown in Fig. 5, the addition of 5 mM vanadate to the normal-calcium ASW induced a small but noticeable and reversible prolongation of the flash response for test flashes just bright enough to elicit a reliable response. Prolongation appeared to arise both from extra waves as shown in the first, second, and last records of column *B* in Fig. 5 and from complex events (column *B*, fourth record) occurring late in the response. This prolongation

is reflected in the averaged waveforms, which are illustrated in Fig. 6. In a second cell examined by the same procedure, we could not be sure of prolongation in the raw records, and we saw only a hint of it in the averages. A third cell, exposed to a slightly lower concentration of vanadate (2.5 mM), showed a definite induction of discrete waves by vanadate but no prolongation of the average response. This lack of prolongation is equivalent to the linear summation of light-induced and drug-induced waves considered in the previous section. Thus, prolongation of the light response can occur in normal-

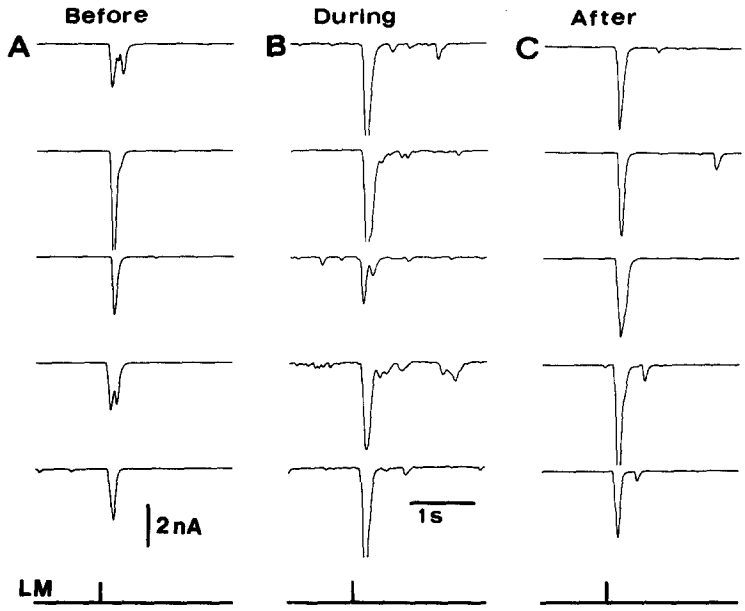


FIGURE 5. Prolongation of flash responses after exposure to 5 mM vanadate in normal-calcium ASW (column *B*). Sequential records of control responses taken before and after exposure to vanadate are shown in columns *A* and *C*. The light monitor (LM) in the bottom row indicates the occurrence of a 20-ms test flash of log intensity −5.8. The averaged waveforms of the entire 50 flash sequences for each condition are shown in Fig. 6. Note that some of the records illustrated here go off the scale of the chart recorder.

calcium ASW, but its occurrence is not tightly linked to discrete-wave induction by vanadate in the dark.

Prolongation by Vanadate in Low-Ca^{2+} ASW

In search of conditions for more reproducible prolongation than just described, we lowered the calcium concentration of the ASW to 1 mM. In the second cell mentioned above (but not shown), which gave only a tiny prolongation with 5 mM vanadate in normal-calcium (10 mM) ASW, we found a much stronger prolongation of the response with 5 mM vanadate in low-calcium (1 mM) ASW (see Fig. 7). Again, prolongation appears to result both

from extra waves (column *B*, first and last records) and complex events (column *B*, third record) occurring late in the response. Five cells examined under these conditions (5 mM vanadate in low-calcium ASW) all exhibited definite prolongation of the light response. Measurements of average waveform parameters of the responses from these cells are given in Table I.

In 5 mM vanadate under reduced calcium conditions, we find from Table I a large, consistent, and reversible prolongation of the flash response of the cell. Prolongation results from a nearly threefold lengthening of the decay time of the average response. Other changes in the response waveform are much smaller and are not consistent from cell to cell. Note that there is no consistent change in the peak amplitude of the flash response. Any decreases in the latency or rise time are small and do not occur consistently in all five cells.

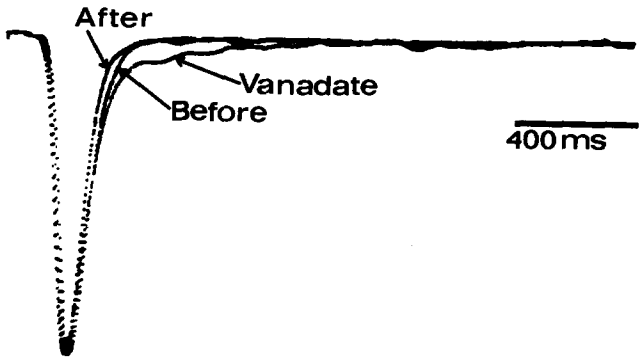


FIGURE 6. Normalized averaged waveforms of flash response currents taken before, during, and after exposure to 5 mM vanadate in normal-calcium ASW as described in Fig. 5. Note the small but distinct shoulder on the falling (late) phase of the response waveform in the presence of vanadate. The averaged peak currents taken before, during, and after exposure to vanadate were 1.4, 0.9, and 1.2 nA, respectively. The decay times (95–5%) of the waveforms taken before, during, and after vanadate were 146, 352, and 140 ms, respectively.

Prolongation after GTP-γ-S Injection in 10 mM Ca^{2+} ASW

Having made these observations on the nature of prolongation with vanadate, we wanted to know whether we could demonstrate prolongation of the light responses by a hydrolysis-resistant analogue of GTP in normal-calcium ASW. As shown in Fig. 8, a cell injected with GTP-γ-S gives prolonged flash response currents under voltage clamp. Prolongation, therefore, results when cells are treated with fluoride, vanadate, or GTP-γ-S. Furthermore, prolongation is not dependent on changes in membrane potential, since it occurs under voltage clamp and it is not altogether dependent on a reduced calcium concentration. As the effects of GTP-γ-S are not reversible (Corson and Fein, 1983), we did not wait for recovery of the cell from GTP-γ-S.

To demonstrate prolongation unequivocally, we averaged the sequence of

responses from which the records in Fig. 8 were taken (see Methods). The normalized average waveforms, which are given in Fig. 9, showed prolongation of the response after injection of GTP-γ-S, as expected from the individual records shown in Fig. 8. However, there are, remarkably, no other appreciable changes in the response waveform. We injected a total of seven cells with GTP-γ-S using the procedure described for Figs. 8 and 9. Although all seven gave evidence of an increased discrete-wave frequency caused by

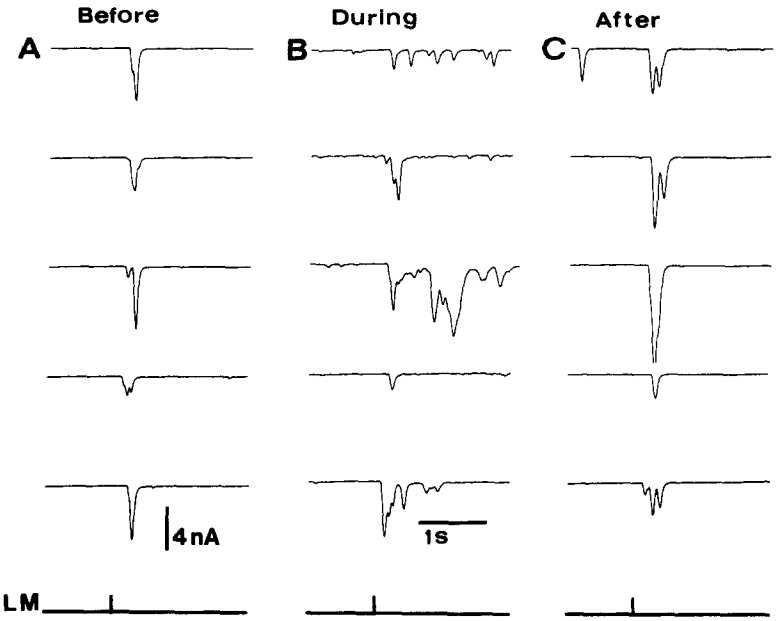


FIGURE 7. Prolongation of flash responses during exposure to 5 mM vanadate in low-calcium (1 mM) ASW (column *B*). Sequential records of control responses taken before and after exposures to vanadate are shown in columns *A* and *C*. The light monitor (LM) on the bottom row indicated the occurrence of a 20-ms test flash of log intensity −5.5. Note the enhanced prolongation of the flash responses (*B*) in low-calcium ASW as compared with normal-calcium ASW (Fig. 5*B*), keeping in mind that the two groups of data are from different cells. These records are from those used to determine the average response for cell 5 of Table I.

the GTP-γ-S injection, only five showed a clearly noticeable shoulder (prolongation) on the falling phase of the average response. Therefore, prolongation is not tightly linked to discrete-wave induction by GTP-γ-S.

DISCUSSION

Intervals Between Discrete Waves

For waves evoked by steady light, conformity to the exponential interval distribution and a lack of correlation among waves are requirements of the hypothesis that one photon produces, at most, one discrete wave. No corre-

lations among waves or exceptions to the exponential interval distribution for light-induced discrete waves have been found for any photoreceptors (for some recent observations, see Yeandle and Spiegler, 1973; Weiss and Yeandle, 1975; Lillywhite, 1977), and this evidence supports the notion that an effectively absorbed photon elicits only one discrete wave. We have reconfirmed these observations in both normal- (10 mM) and low- (1 mM) calcium ASW (Figs. 1 and 3). Furthermore, we have shown that the addition of

TABLE I

Waveform parameter	Experimental condition	Cell number 1	2	3	4	5	Change*	Percent change
Peak current (nA)	Control before	12.3	8.3	6.2	3.4	2.8		
	Vanadate	14.8	6.4	12.6	5.6	3.4	+1.3±2.0	+18
	Control after	15.8	3.6	9.2	7.9	3.2		
Latency (ms)	Control before	154	158	130	146	140		
	Vanadate	128	146	108	126	146	−16±10	−11
	Control after	158	170	130	130	156		
Rise time (ms)	Control before	88	98	100	90	110		
	Vanadate	88	98	86	74	108	−12±6	−12
	Control after	100	120	100	102	120		
Decay time (ms)	Control before	292	216	326	338	188		
	Vanadate	942	734	574	1,782	1,452	+818±496	+293
	Control after	312	304	224	342	246		

Waveform parameters from the averaged flash response waveform in five cells exposed to 5 mM vanadate in low-calcium (1 mM) ASW. The latency was measured from the leading edge of the test flash to the time required for the response to reach 5% of its final magnitude in the downward direction. Rise times and decay times were measured between the 5 and 95% levels of response amplitude. The values of the parameters in vanadate or in control solutions before and after vanadate exposure are given by v, cb, and ca, respectively. The mean and standard deviation of the change ($v - cb/2 - ca/2$) and the change as a percentage of the average control values ($cb + ca$)/2 are given in the last two columns on the right, respectively. * Change = ($v - cb/2 - ca/2$) ± SD.

fluoride to normal-calcium ASW can induce the production of uncorrelated discrete waves that exhibit an interval histogram that appears to be exponential (Fig. 1). The lack of correlation and the exponential interval distributions of both spontaneous and fluoride-induced discrete waves are consistent with the notion that these waves arise independently from a random molecular process within the cell.

Although discrete waves appear to have an exponential interval distribution

under a variety of conditions, we have found that the exponential distribution of intervals (Figs. 2*C* and 3*C*) does not hold in the dark under the special conditions of simultaneous exposure to moderate concentrations of fluoride and reduced concentrations of external calcium. Under these conditions, bursts of waves appear in the records and correlations appear among waves. Similar and sometimes more dramatic bursts than those shown in Fig. 2*C* are

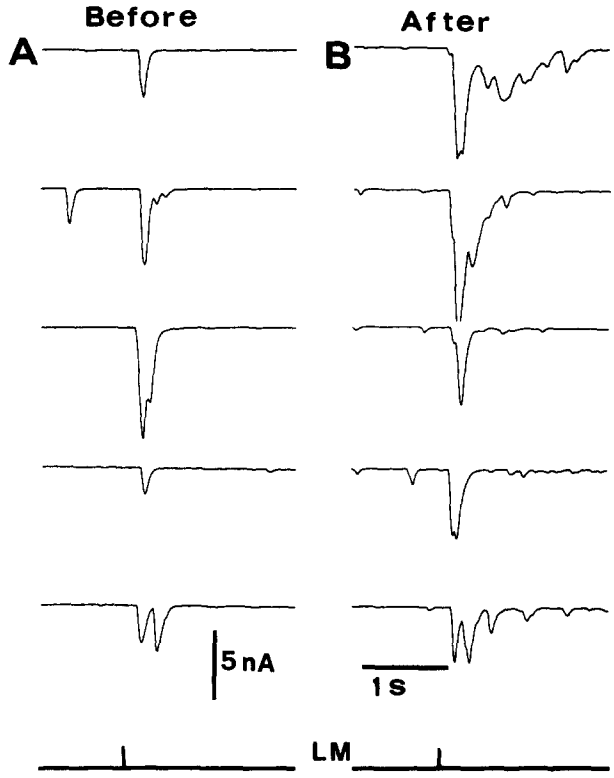


FIGURE 8. Prolongation of flash responses after injection of GTP-γ-S into a cell bathed in normal-calcium ASW. Sequential records taken before and after injection are shown in columns *A* and *B*, respectively. At the bottom, the light monitor (LM) indicates the time of occurrence of a 20-ms test flash of log intensity −6.0. Prolongation was elicited by a steady 10-min injection of GTP-γ-S at 1 nA. The averaged waveform corresponding to these responses is shown in Fig. 9. Note that the second record in *B* went off the scale of the chart recorder.

also found when cells treated with vanadate (Fig. 4*A*) or GTP-γ-S (results not shown) are exposed to reduced external calcium. Sometimes bursts are observed on exposure of cells to high concentrations of drugs alone in normal-calcium ASW. Because we have observed bursts of waves in rare, untreated cells (Fig. 4*B*) and in recordings from cells after exposure to very bright lights (Fig. 4*C*), we suggest that bursts may be natural phenomena that are

greatly accentuated by fluoride and the other drugs. Clearly, bursts cannot be the result of a simple Poisson process. We suggest that they are the result of a process in which activation of a single molecular site gives rise to a variable number of waves. From the nature of the compounds that elicit discrete waves and bursts in the dark, we suspect that the active site is a GTP-binding protein, N, which normally helps to turn off the production of both spontaneous and light-evoked waves. However, none of the results obtained so far actually link the bursts observed in the dark to the prolongation of the light response, which we consider in the following section.

Prolongation of the Light Response

In low-calcium ASW, vanadate reliably prolongs the response to test flashes that are just bright enough to elicit a response in ventral photoreceptors.

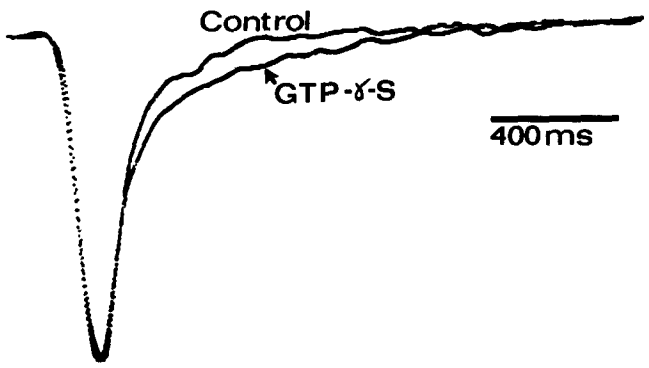


FIGURE 9. Normalized averaged waveforms of flash response currents before and after injection of GTP-γ-S as described in Fig. 8. The decay time (95–5%) of the waveform was prolonged to 818 vs. 410 ms in the control. The average peak current after GTP-γ-S injection (4.6 nA) was not appreciably different from the control peak current (4.4 nA). Except for the prolonged decay, the waveforms are similar.

The principal effect on the averaged response waveform in both normal- and low-calcium ASW is the induction of a shoulder on the falling phase of the response waveform (Fig. 6 and Table I). There is little effect on the initial phase of the response. Prolongation can be elicited by fluoride (Fein and Corson, 1979) and by GTP-γ-S (Figs. 8 and 9), as well as by vanadate. We conclude that these drugs, which induce discrete waves in ventral photoreceptors in the dark, also interfere with the normal process of phototransduction at low flash intensities by prolonging the light response. In individual records, the shoulder of prolongation arises from what appear to be extra discrete waves late in the response, or from complex, long-lasting events associated with the flash response (Figs. 5, 7, and 8).

Before we consider the mechanism of prolongation, we wish to mention several points concerning prolongation. First, we have tried to show that prolongation can definitely be elicited under restricted conditions (low cal-

cium) but that it can be induced, albeit less reliably, under normal conditions (normal-calcium ASW). Second, since prolongation is not tightly linked to the induction of discrete waves, we cannot be certain that the two phenomena arise by exactly the same process. Third, we note that another type of prolongation occurs with exposure to brighter lights. Whereas the prolongation we observed with dim test flashes decays within seconds after the flash, Bolsover and Brown (1980) observed a prolonged increase in the frequency of discrete-wave occurrence after injection of GMP-PNP (guanosine-imidotriphosphate) and exposure to bright light. Such a prolongation may represent a light-induced increase in the availability of GTP-binding sites to the GTP analogue, which would otherwise slowly become available in the dark (see Corson and Fein, 1983). Fourth, the effect of calcium on the number of waves in the light response suggests that calcium may act at more than one stage of transduction.

There are several observations concerning the role of calcium in transduction. (*a*) Previous work with calcium in *Limulus* ventral photoreceptors has revealed that removal of extracellular calcium lengthens the time to peak of the light response in dark-adapted cells (Lisman, 1976). Martinez and Srebro (1976) reported that reducing extracellular calcium increased the latency of discrete waves, but did not affect the efficiency of discrete-wave production. They further reported that reducing extracellular calcium increased the amplitude of small discrete waves in some cells but not in those with the largest discrete waves. (*b*) A rise in intracellular calcium has been proposed to mediate the process of light adaptation in ventral photoreceptors (Lisman and Brown, 1972). Evidence for this hypothesis has recently been summarized by Fein and Szuts (1982). Light adaptation is accompanied by a reduction in latency and amplitude of the light response. (*c*) Finally, there is the enhancement of prolongation in low calcium reported here. We suggest that the enhancement of prolongation in low calcium occurs at a different stage of transduction from the site responsible for the other known effects of calcium because (*a*) prolongation in low calcium appears to result from an alteration of the number of discrete waves in the response without appreciable changes in the response amplitude or latency, whereas (*b*) the other known effects of Ca are associated with changes in amplitude and latency, but not the number of waves in the response.

What Is the Mechanism of Prolongation?

Prolongation of the light response does not appear to result from a general increase in latency or a simple slowing of the kinetics of discrete waves. Figs. 6 and 9 and Table I show that GTP-γ-S and vanadate have no substantial effects on the latency or rise time of the average response, and many individual waves have approximately normal time courses.

Prolongation does not appear to result from a simple increase in the number of photoactivated rhodopsin molecules (Rh*) because we found no drug-induced change in the amplitude of the average response. Suppose that the number of Rh* was raised by increasing the flash intensity. Lisman and

Brown (1975) have shown that at low flash intensities, the amplitude of evoked currents under voltage clamp is linearly related to flash intensity. Conversely, Lisman and Strong (1979) have shown that when the flash intensity is constant, as in our experiments, there is an approximately linear relationship between the response amplitude and the concentration of rhodopsin. In both cases, an increase in the number of Rh* results in an increase in response amplitude. We did not find any consistent increase in the peak amplitude of the flash response during exposure to vanadate or GTP-γ-S.

Although we cannot rule out other alternatives, such as the unlikely possibility that the inhibitors selectively raise the number of Rh* producing only waves of long latency, we suggest that the most likely explanation of prolongation is that the inhibitors allow or facilitate the production of complex events and muliple discrete waves by single photoactivated rhodopsin molecules.

The most desirable test of this multiple-wave hypothesis would be to compare the numbers of waves in responses to the expected numbers calculated from simple Poisson statistics by using the fraction of flashes producing no response (Weiss and Yeandle, 1975; Lillywhite, 1977; cf. Del Castillo and Katz, 1954). We have been unable to apply this test because we could not effectively count the number of null responses in our experiments because of the presence of a high rate of drug-evoked waves in the dark. However, prolongation of the light response is not tightly linked to activity in the dark, and in the future it may be possible to find conditions that selectively suppress dark activity and thereby make it possible to count the number of failures of the response.

A Biochemical Interpretation of Prolongation

In the accompanying paper (Corson and Fein, 1983), we have suggested that hydrolysis of GTP participates in turning off the light response once it has been initiated by rhodopsin. Prolongation of the light response by fluoride, vanadate, and GTP-γ-S is consistent with this hypothesis. Investigation of the average test flash response has shown that vanadate and GTP-γ-S prolong the decay of the light response while having little or no effect on the latency, rise time, and peak amplitude of the response. The phosphatase inhibitors appear to be selective in that they do not interfere with the initiation of the light response but rather inhibit the inactivation or turning off of the response. As we have already suggested, this inhibition of the turn-off reaction may manifest itself in the production of multiple waves and complex events arising from a single photoactivated rhodopsin.

The putative turn-off reaction may occur by hydrolysis of GTP at a GTP-binding site analogous to that which regulates adenylate cyclase (Fein and Corson, 1981), or it may occur by a different mechanism involving nucleotide hydrolysis at some other kind of enzymatic site. The latter, more general alternative has also been considered by Payne (1982) in an analysis of the effects of fluoride on locust photoreceptors. We are currently seeking to identify the site of action of the agents that induce discrete waves and prolong

the light response. If the site is consistent with the model proposed earlier (Corson and Fein, 1983), we hope to find a light-activated GTPase that is separate from rhodopsin. We are encouraged in this pursuit by the reports of a light-activated GTPase in the photoreceptors of the cephalopod eye (Calhoon et al., 1980; Vandenberg and Montal, 1982).

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