Chemical Excitation of *Limulus* Photoreceptors

I. Phosphatase Inhibitors Induce Discrete-Wave Production in the Dark

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ABSTRACT Molybdate, tungstate, fluoride, vanadate, and GTP-γ-S [guanosine-5′-0-(3-thiotriphosphate)] were injected into *Limulus* ventral photoreceptors by ionophoresis from microelectrodes. All of these drugs induce discrete waves of depolarization similar in waveform to, but smaller in amplitude than, those normally elicited by dim light. As for light-evoked waves, the amplitude of drug-induced waves decreases with light adaptation. For the compounds examined so far (fluoride, vanadate, GTP-γ-S), the drug-induced waves share a reversal potential with light-induced discrete waves at about +15 mV. The induction of discrete waves by fluoride, vanadate, and molybdate was found to be reversible, whereas the induction of waves by GTP-γ-S was not. Unlike fluoride and vanadate, which induce waves when added to the bath, molybdate appears to be ineffective when applied extracellularly. Because of the similarity of the drug-induced waves to light-induced discrete waves, we conclude that the drug-induced waves arise from a process similar or perhaps identical to visual excitation of the photoreceptor. However, the smaller size of drug-induced waves suggests that they arise at a stage of phototransduction subsequent to the isomerization of rhodopsin. On the basis of the chemical properties and action of the drugs, we suggest that discrete waves may arise through the activation of a GTP-binding protein.

INTRODUCTION

In *Limulus* photoreceptors the light response arises from the summation of discrete waves of depolarization (Dodge et al., 1968; Wong, 1978). Consequently, the biochemical events that underlie discrete-wave production are fundamental to the process of visual transduction. For some time we have

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sought to probe the molecular events of visual transduction by pharmacological means using discrete-wave production as an assay for visual excitation.

Recently (Fein and Corson, 1979, 1981*b*), we reported that fluoride, vanadate, and GTP-γ-S induce the production of discrete waves in the absence of light. Fluoride and vanadate are both phosphatase inhibitors (Hewitt and Nicholas, 1963) and GTP-γ-S inhibits GTPase (phosphatase) activity in turkey erythrocytes (Selinger and Cassel, 1981). Following these experiments, we sought to determine whether related phosphatase inhibitors (see Hewitt and Nicholas, 1963) could also induce the production of discrete waves, and if so, whether they could suggest if their site of action was intracellular or not. To this end, we tested whether molybdate and tungstate induce the production of discrete waves when applied either extracellularly or intracellularly. We report here that intracellular injection of either molybdate or tungstate ions induces the production of discrete waves in *Limulus* photoreceptors, but that molybdate is ineffective when applied extracellularly.

Next, we wanted to know whether the discrete waves induced by the initial three compounds shared a common ionic mechanism with the light-induced discrete waves. To this end, we compared the reversal potentials of the two kinds of discrete waves. In ventral photoreceptors, the light response results from an increase in membrane conductance (Millecchia and Mauro, 1969*b*). The reversal potential of the ionic currents arising from this conductance increase has a nominal value of +15 mV (Millecchia and Mauro, 1969*b*). We report that the reversal potential for discrete waves elicited by fluoride, vanadate, and GTP-γ-S is the same as the reversal potential of light-induced discrete waves. This finding is consistent with the hypothesis that there is a common ionic mechanism for the light-induced and drug-induced discrete waves.

We then sought to compare the distribution of amplitudes of drug-induced waves with the amplitude distributions of light-evoked and spontaneous waves. In *Limulus*, spontaneous waves have been found to be smaller on the average than light-evoked waves, and this finding led Yeandle and Spiegler (1973) to suggest that spontaneous waves arise at a molecule other than rhodopsin. We report that drug-induced waves are also smaller on average than light-evoked waves. Finally, we suggest a molecular model of phototransduction to account for these findings.

A preliminary account of some of these results has appeared in abstract form (Fein and Corson, 1981*a*).

METHODS

The techniques for stimulating and recording from *Limulus* ventral photoreceptors have been described in detail elsewhere (Fein and Charlton, 1975, 1977*a*), as has the composition of the artificial seawater (ASW) (Fein and Corson, 1979). Our methods are similar to those originally described by Millecchia and Mauro (1969*a*, *b*).

The test substances were applied either extracellularly by addition to the ASW bathing the preparation or intracellularly by ionophoretic injection from pipettes. Drugs were ionophoretically injected into cells by passing 1-nA currents through

electrodes containing the drugs at 100-mM concentrations in distilled water. Sodium tungstate, sodium vanadate (meta), and potassium fluoride were obtained from Fisher Scientific Co. (Fair Lawn, NJ). Sodium molybdate was obtained from Sigma Chemical Co. (St. Louis, MO). GTP-γ-S was obtained from Boehringer-Mannheim (Indianapolis, IN). The pH of the injection solutions of the nucleotide was adjusted to within 0.5 pH unit of pH 7.0 by the addition of KOH. Microelectrodes filled with 100 mM KF developed very high resistances and would not pass current when we tried to eject fluoride from them by ionophoresis. Fluoride could be ejected from these pipettes after adjustment of the pH of the injection solution to 6.5 with hydrofluoric acid.

All of the salts of inorganic compounds used in this study could be ionophoresed from pipettes, as expected from their dissociation constants (Dean, 1979). GTP-γ-S could also be ionophoresed from pipettes, and it appears to have dissociation constants close to those of GTP, as indicated by its electrophoretic mobility (Goody and Eckstein, 1971). We take 1.0 to be an upper limit on the transport numbers for all of these compounds. The actual transport numbers are probably smaller, but they vary from compound to compound. Although it might be of some peripheral interest here, we did not attempt to determine a transport number for molybdate because of its complex polyionic chemistry in solution (see Van Etten et al., 1974).

Reversal potentials of light-induced and drug-induced discrete waves were determined in the following manner. Cells were impaled with two microelectrodes and allowed to dark adapt. Drug-induced waves were then elicited by the addition of fluoride or vanadate to the bath or by ejection of GTP-γ-S from the voltage-sensing electrode. When the rate of induced activity was sufficiently high, the cell was clamped to its resting membrane potential, and test flashes just bright enough to evoke a reliable response were given at 40-s intervals. The membrane potential was then moved in 10-mV steps through the reversal potential by a series of 20-s-long command pulses, each beginning 10 s before the test flash. 5 s before the test flash, the large steady state component of the transmembrane current resulting from the voltage step was sampled and subtracted from the current records to facilitate the display of the small discrete-wave currents during a 10-s interval containing the flash. Voltage-clamp records were made in low-calcium (1 mM Ca^{2+}) ASW to avoid desensitization produced by voltage-dependent Ca^{2+} entry (O'Day et al., 1982).

To introduce consistency into the measurement of discrete waves, a discriminator with a band-pass input (10–80 Hz) and a 0.05-nA (or 0.5-mV) threshold was used to mark their occurrence. This device yielded counts similar to those obtained by manual analysis of the records and gave accurate counts up to frequencies of 5 waves/s. To measure the rate of occurrence of waves, we counted the number of waves in 200-s intervals.

To separate out the contribution of spontaneous waves to the amplitude distributions of light-evoked and fluoride-induced waves, and to compensate for small differences in the rates of occurrence of the various waves during a fixed time interval, we subtracted the amplitude histogram of spontaneous waves from the amplitude histograms generated in the presence of light or fluoride and then normalized all three histograms to have unit areas as if the spontaneous discrete waves and light- and fluoride-induced increments occurred separately and at identical rates. In doing this, we assumed that light-induced and fluoride-induced discrete-wave occurrences were superimposed on the spontaneous background rate. Because voltage-dependent conductances modify the voltage amplitudes of light-induced discrete waves, the amplitudes of discrete-wave currents were measured in cells voltage-clamped to their resting membrane potentials.

RESULTS

Chemically Induced Discrete Waves

We have previously reported that fluoride, vanadate, and GTP-γ-S induced the production of discrete waves in the absence of light (Fein and Corson,

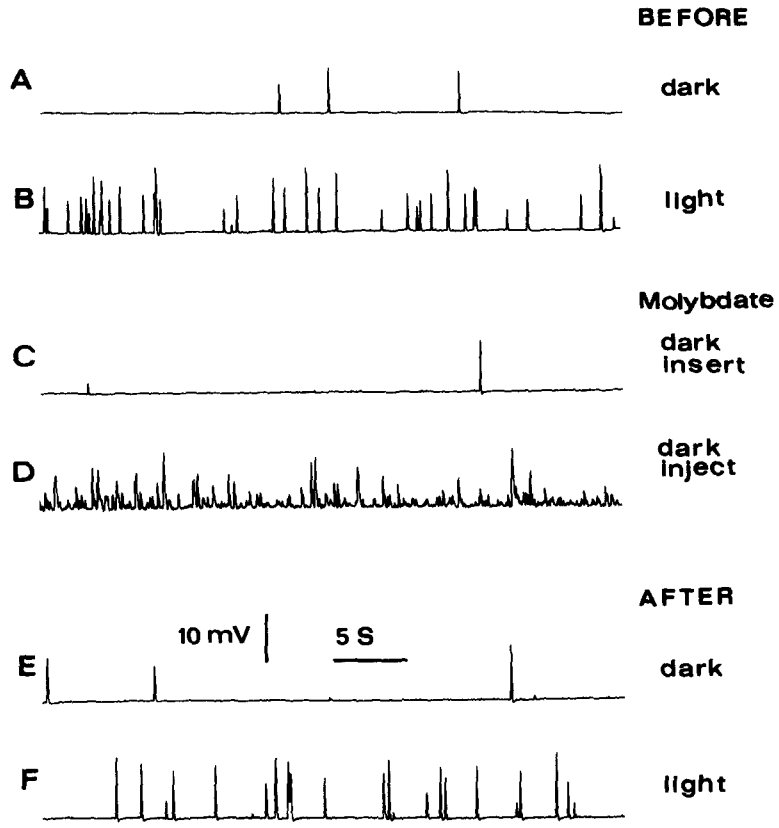

FIGURE 1. Induction of discrete waves by molybdate injection. Control recordings of spontaneous and light-induced discrete waves are shown in records *A*, *B*, *E*, and *F*. The steady light stimulus in records *B* and *F* was attenuated 7.7 log units below the maximum intensity available at the source. Between records *B* and *C*, a second electrode containing 100 mM sodium molybdate was inserted into the cell. Between records *C* and *D*, molybdate was injected by 1-nA hyperpolarizing current pulses of 5 s duration at 10-s intervals for 5 min. A negligible depolarization of ~1 mV accompanied insertion of the second electrode and injection of molybdate. Records *E* and *F* were taken 45 min after withdrawal of the molybdate electrode.

1979, 1981*b*). In Figs. 1 and 2 we show that intracellular injection of molybdate and tungstate in the dark also induces discrete waves that have a waveform remarkably similar to light-induced discrete waves. Note in Fig. 1 that the molybdate-induced waves are smaller in amplitude than those elicited by light. The same is also true for the tungstate-induced waves, although this

is not clearly illustrated in the records of Fig. 2. We have already shown that vanadate and GTP-γ-S induce discrete waves when injected, and for completeness, we injected two cells (not shown) with fluoride (see Methods). Both exhibited a dramatic rise in the frequency of small discrete waves. Thus, when injected intracellularly, all five chemicals (fluoride, vanadate, GTP-γ-S, molybdate, and tungstate) induce discrete waves that have a waveform similar to, but on average an amplitude smaller than, those induced by light.

As shown in Fig. 1, the effect of a single molybdate injection is reversible after ~45 min. The effects of tungstate persist for hours, and we did not pursue reversibility in this case. We have previously reported that the effects of fluoride (Fein and Corson, 1979) and vanadate (Fein and Corson, 1981*b*)

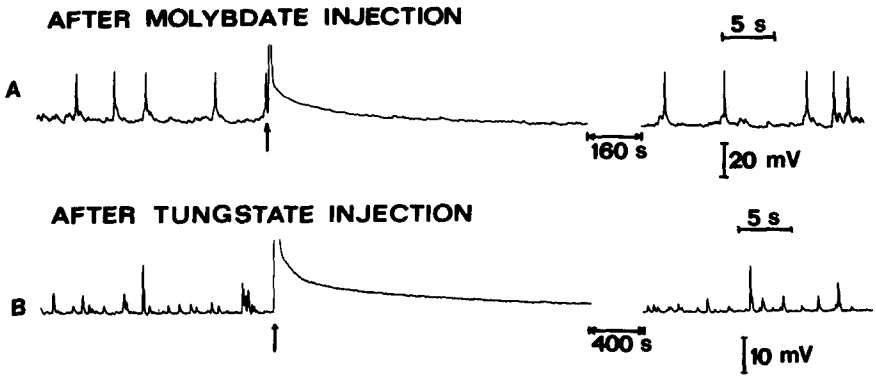

FIGURE 2. Light adaptation of drug-induced discrete waves. The recordings in *A* and *B* were made after discrete waves were induced by injection of molybdate and tungstate, respectively. At the arrow, a bright 20-ms adapting flash caused the temporary attenuation of the drug-induced waves. After the adapting flash, the ongoing drug-induced discrete waves gradually recovered in amplitude until they regained the amplitude they had before the adapting flash. The intensity of the adapting flash was attenuated 3 log units below the maximum intensity available from the light source. Portions of each record were deleted from the original continuous records for convenience of illustration of recovery. Note that the photoreceptor's response to the adapting flash goes off scale.

were reversible. In contrast, the effects of GTP-γ-S injection did not appear to be reversible over the course of ~10 h in several cells examined for evidence of reversibility.

Light Adaptation of Drug-induced Discrete Waves

In addition to their characteristic waveform, susceptibility to light adaptation is a principal property of light-induced discrete waves—they are temporarily diminished in amplitude after a bright adapting flash. Discrete waves elicited by molybdate and tungstate also undergo light adaptation, as shown in Fig. 2. We previously reported that discrete waves induced by fluoride (Fein and Corson, 1979), vanadate, and GTP-γ-S (Fein and Corson, 1981*b*) undergo adaptation in a similar fashion.

Is There an Extracellular Site of Action?

Unlike fluoride and vanadate, molybdate does not activate receptors when applied externally. Four cells were exposed to a minimum of 5 mM molybdate in the ASW for at least 40 min. None exhibited an obvious increase in discrete-wave activity. One of the four cells was exposed to 30 mM molybdate for 50 min and showed no increase in discrete-wave activity. However, a second cell from the same nerve showed a noticeable increase in discrete-wave activity after molybdate injection (35 2-s current pulses at 1 nA). An estimate of the upper limit of the effective intracellular concentration of molybdate for this cell can be obtained from the injection as follows. If we assume (*a*) a typical cell volume of 250 pl, (*b*) negligible diffusion leakage of molybdate from the pipette, and (*c*) that all the charge is carried by molybdate, then the effective intracellular concentration of molybdate calculated from the net charge transfer was 3 mM. This concentration was an order of magnitude smaller than the external concentration of molybdate applied to the other cell from the same nerve. Thus, it is likely that the only site of action for molybdate is within the cell.

Common Reversal Potentials for Light-induced and Drug-induced Waves

The discrete waves induced by fluoride, vanadate, and GTP-γ-S share a common reversal potential with light-induced discrete waves, as illustrated in Fig. 3. To compare the reversal potentials of light-induced and drug-induced discrete waves, we followed the procedure described in Methods. As can be seen in the records of Fig. 3, the reversal potentials for the discrete waves induced by light are the same as the reversal potentials of the discrete waves induced by each of the three drugs and are approximately +15 mV. Reversal potential measurements were replicated in three separate cells for each of the three drugs. Reversal potentials for drug-induced and light-induced discrete waves did not differ in any instance.

Amplitude Distributions of Discrete Waves

The shape of discrete waves elicited by drugs closely resembles the shape of light-induced and spontaneous discrete waves, as shown in Figs. 1–3. In records such as these, one's attention is drawn to the larger drug-induced discrete waves, which are clearly comparable in amplitude to light-induced discrete waves. However, since many smaller waves occur in the records, the distribution of discrete-wave amplitudes provides a more accurate means for comparison of the amplitudes of the drug-induced, light-evoked, and spontaneous discrete waves.

To make these comparisons, it is necessary to have stable conditions under which to measure the amplitude distributions. With intracellular injection of drugs, we were unable to find such conditions. Therefore, we turned to drugs that could be applied extracellularly. Fluoride sufficed for these purposes, and appropriate conditions were determined by measuring the time course of the induction of discrete waves for various concentrations of fluoride. For this determination, we monitored the rate of discrete wave

occurrence, as described in Methods. The results of one such experiment are given in Fig. 4. On exposure to low concentrations of fluoride (2.5, 5.0 mM; Fig. 4), discrete-wave frequencies rose slowly and eventually stabilized after 20–30 min. At the higher concentration (7.5 mM; Fig. 4), the discrete-wave

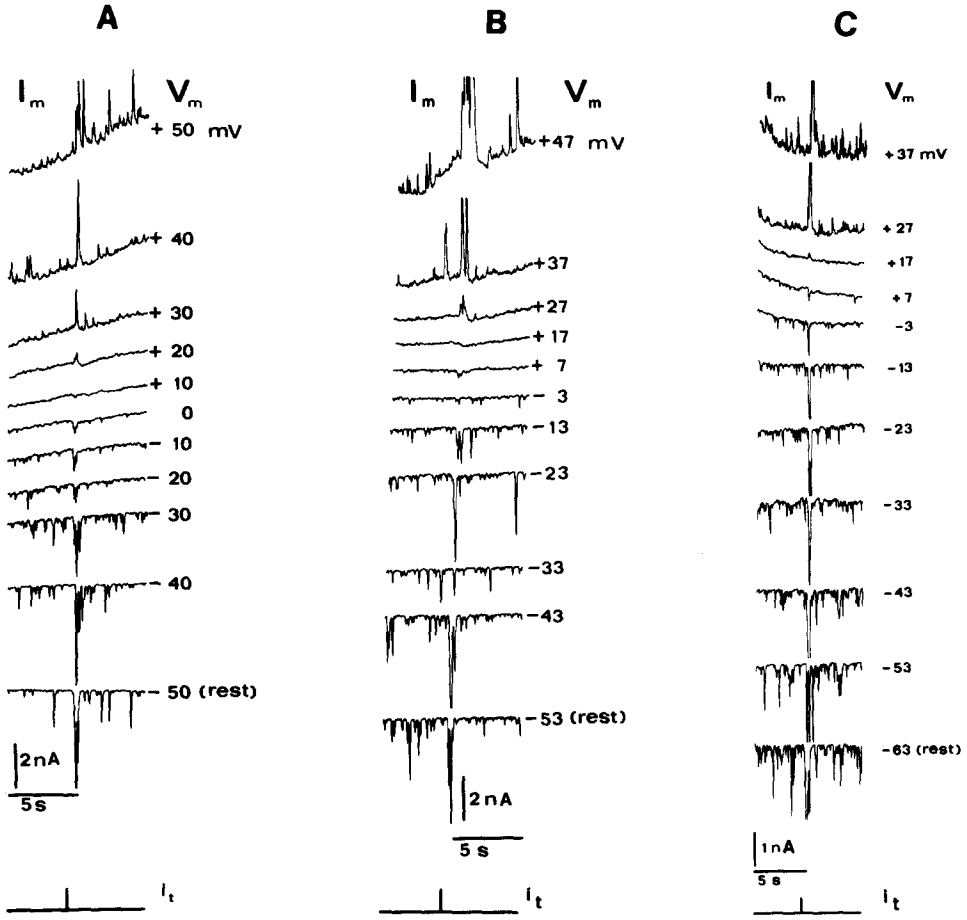

FIGURE 3. Reversal potentials for discrete waves induced by fluoride (*A*), vanadate (*B*), and GTP-γ-S (*C*) were obtained as described in Methods. Flashes are indicated by the test flash monitor (i_t) at the bottom of each column. Reversal potentials for drug-induced and light-induced discrete waves are the same in each case and occur at approximately +15 mV. The values of the clamped membrane potential are indicated to the right of the current traces. The 20-ms test flashes were attenuated either 5.5 (*A* and *B*) or 4.5 (*C*) log units from the maximum available intensity. Note that on this expanded current scale, the response to the test flash sometimes goes off scale.

frequency did not stabilize over the 1-h exposure period. In all cases the cell recovered after the fluoride was washed from the bath. In a series of experiments similar to that shown in Fig. 4, we found that the elevation in

discrete-wave frequency is relatively stable and usually reversible for applications of fluoride in the range of 1–6 mM.

The distributions of amplitudes (see Methods) of spontaneous, light-induced, and fluoride-induced discrete waves overlap considerably but are not identical, as can be seen in Fig. 5. Fluoride-induced and spontaneous discrete waves are smaller than light-induced discrete waves. The modal value for light-induced discrete waves is 0.4 nA (see Fig. 5*B*). Because the modal value for fluoride-induced and spontaneous discrete waves occurs below the unit

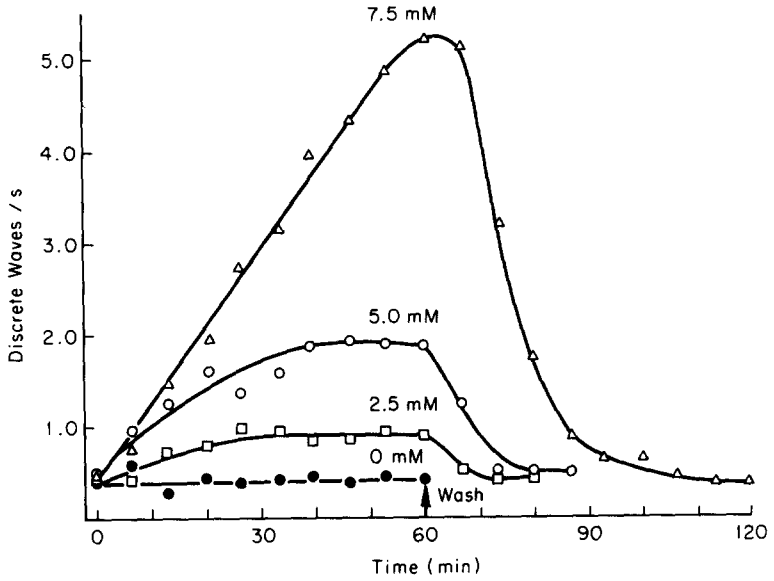

FIGURE 4. Time course and dose dependence of the effect of fluoride on discrete-wave frequency from one cell. Stability of the spontaneous (untreated) discrete-wave frequency was measured (see Methods) over the course of 1 h (0 mM). The cell was then exposed to successive concentrations of fluoride for 1 h each, starting at zero time. Between exposures, the cell was washed just long enough to allow the discrete-wave frequency to return to the control level. The discrete-wave frequency reaches a quasi-steady level at the lower concentrations (2.5 and 5 mM) of fluoride but not at the higher one (7.5 mM). The frequencies of occurrence plotted here were determined as described in Methods. The solid curves were drawn through the data points by eye.

of resolution (0.1 nA), these waves are on the average less than one-quarter of the size of light-induced discrete waves. The proportion of small spontaneous discrete waves that we observed is similar but more pronounced than the proportion of small waves reported in a similar experiment carried out in organ culture medium instead of ASW (Bayer and Barlow, 1978).

During exposure to fluoride in the experiment shown in Fig. 5, the average amplitude of 10 responses to 20-ms test flashes of log relative intensity −5.5 was 2.9 ± 1.4 nA (x ± SD) and did not differ appreciably from the response

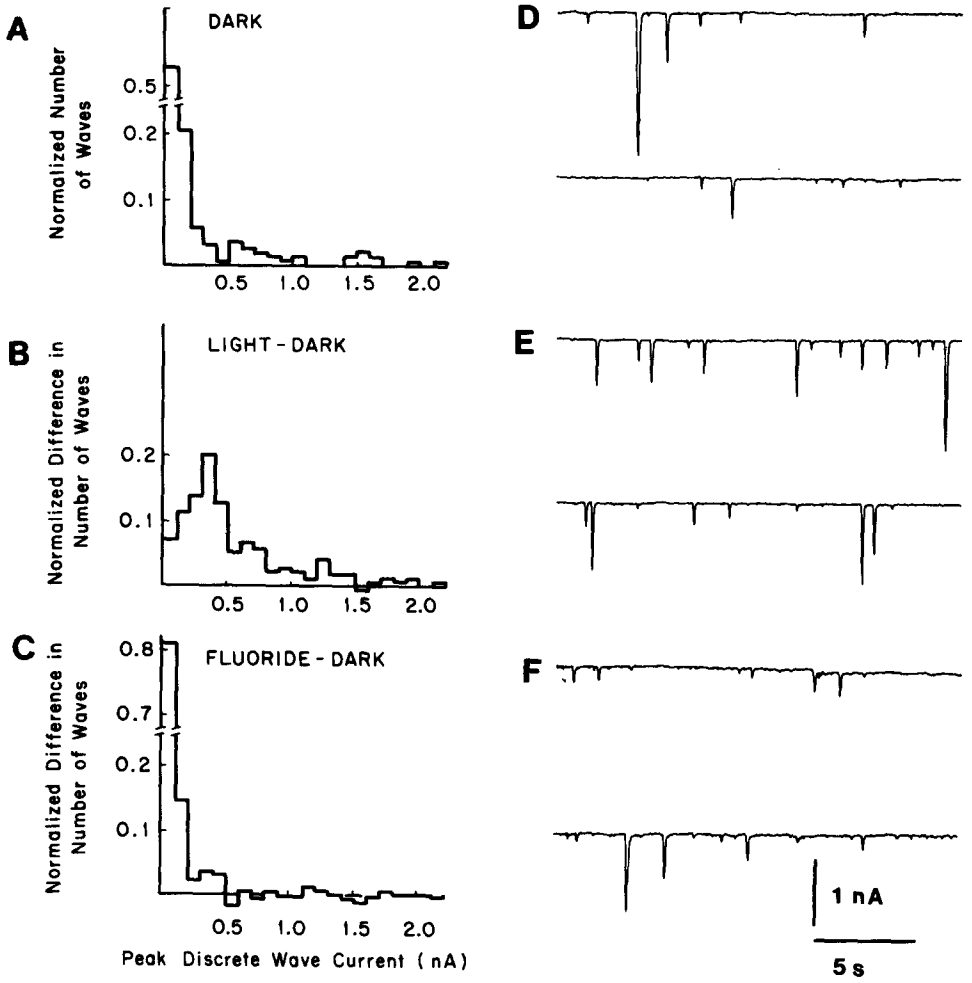

FIGURE 5. Normalized amplitude histograms of spontaneous discrete waves (*A*) and the increments evoked by light (*B*) and by fluoride (3.75 mM) (*C*), with the sample records from which the histograms were constructed (*D–F*) appearing opposite each histogram. For graph *A*, the amplitudes of 166 spontaneous discrete waves occurring in a 500-s interval are histographed on a normalized scale with a unit area per wave of 1/166. The corresponding spontaneous discrete wave rate in *A* was 166/500 or 0.33 discrete waves/s. For graph *B*, a dim light evoked 362 discrete waves in a 500-s interval. The histogram of the light-induced frequency increment (*B*) was obtained by subtracting the raw spontaneous discrete wave histogram from the raw histogram of the 362 discrete waves and normalizing the remaining histogram to a unit area of 1/(362 − 166). The light-induced frequency increment from *B* was (362 − 166)/500 or 0.39 discrete waves/s. In a similar fashion, the fluoride-induced frequency increment was plotted in graph *C*. The fluoride-induced frequency increment was (397 − 166)/500 or 0.46 discrete waves/s. The amplitude distributions overlap but are not identical for the three kinds of discrete waves.

amplitude of 3.0 ± 1.4 nA measured before exposure. Therefore, the increase in the frequency of occurrence of waves observed in the presence of fluoride is not due to a simple increase in the fraction of waves above the threshold resulting from an increase in the average size of waves. Similarly, the smaller size of the fluoride-induced waves cannot be due to a decrease in the overall amplitude of waves. For the experiment illustrated in Fig. 5, we chose a cell that was isolated from other receptor cell bodies on the nerve to avoid electrontonic coupling of discrete waves from other cells. The increment in discrete-wave frequency induced by fluoride cannot be accounted for by an increase in electrotonic coupling to other cells.

DISCUSSION

Chemically Induced Discrete Waves

Five diverse compounds induce the production of discrete waves in *Limulus* ventral photoreceptors in the absence of light. These include molybdate (Fig. 1) and tungstate (Fig. 2), along with fluoride (Fein and Corson, 1979), vanadate, and GTP-γ-S (Fein and Corson, 1981*b*). The discrete waves induced by all five compounds bear a remarkable resemblance to the discrete waves normally evoked by light. Their similarity to light-induced waves raises the possibility that they arise by activation of the pathway of phototransduction.

If drug-induced discrete waves arise by chemical activation of transduction, they would be expected to share other principal properties of light-induced discrete waves, in addition to the waveform. (*a*) Based on the work of Dodge et al. (1968) and Wong (1978), it appears that light adaptation occurs by an attenuation of the amplitude of light-induced discrete waves, and we have found that the amplitudes of waves induced by both molybdate and tungstate are attenuated by bright flashes (Fig. 2), as are the amplitudes of discrete waves evoked by fluoride (Fein and Corson, 1979), vanadate, and GTP-γ-S (Fein and Corson, 1981*b*). (*b*) Light-induced discrete waves arise by an increased ionic conductance with a reversal potential of approximately +15 mV (Millecchia and Mauro, 1969*b*). If chemically induced discrete waves arise from the same ionic conductance mechanism, they should have the same reversal potential. We found that the reversal potentials for discrete waves induced by fluoride, vanadate, and GTP-γ-S do not differ from the reversal potential of light-induced discrete waves (Fig. 3). From the similarities of the drug-induced discrete waves to light-induced discrete waves so far established, we tentatively conclude that all five of these agents do indeed activate the pathway of phototransduction.

Site of Drug Action

The findings presented in Results show that each of the five chemicals will induce discrete waves when injected intracellularly. This implies that there is an intracellular site of action for all of them. Since all five chemicals are phosphatase inhibitors and since all have essentially the same effect, we

suggest that they probably share a single intracellular site of action. Furthermore, we suggest that those drugs that are effective when applied extracellularly must first enter the cell to have their effect. The evidence against a second extracellular site is as follows: (*a*) Molybdate is ineffective when applied extracellularly (see Results). (*b*) Fluoride (Fig. 4) and vanadate (Fein and Corson, 1981*b*) take tens of minutes to become effective when applied extracellularly, whereas vanadate, when injected intracellularly, induces discrete waves within several seconds (Fein and Corson, 1981*b*). This suggests that vanadate and fluoride must first reach some compartment (probably intracellular) to become effective.

Mechanism of Drug Action

How might such a diverse group of compounds produce excitation in ventral photoreceptors? Fluoride, vanadate, molybdate, and tungstate are all known phosphatase inhibitors (Hewitt and Nicholas, 1963; see also Van Etten et al., 1974), and GTP-γ-S inhibits the GTPase of turkey erythrocytes (see Selinger and Cassel, 1981). The ineffectiveness of ATP-γ-S for injections similar to effective GTP-γ-S injections in the dark indicates that the site in question is selective for GTP over ATP (Corson and Fein, 1981*b*), but the degree of selectivity is not clearly established at this point (see Bolsover and Brown, 1982). The uniformity of action of all five phosphatase inhibitors suggests that GTP hydrolysis may play a role in transduction, but not as a source of energy or phosphate for excitation, since hydrolysis inhibitors produce excitation.

In addition to our experiments with GTP-γ-S, there is a report from Bolsover and Brown (1980) that ventral photoreceptors could be excited by the hydrolysis-resistant GTP analogue GMP-PNP (guanylyl imidodiphosphate), but not by its reaction products (imidodiphosphate, 5′-guanosine monophosphate, and 3′5′-cyclic guanosine monophosphate). They also reported that two periods of bright illumination were sometimes necessary to induce discrete-wave production after the injection of this hydrolysis-resistant analogue (Bolsover and Brown, 1980). We interpret the requirement of illumination after injection of GMP-PNP, together with the persistence of the effect of GTP-γ-S (see Results), to indicate that light enhances the access of hydrolysis-resistant GTP analogues to the site of discrete-wave production and that, once there, they become bound to the now-activated site. However, at this point, we do not know how reversible the effects of GTP-γ-S would be if we could purge the cell of excess GTP-γ-S after activation.

It is unlikely that some nonspecific ionic effect can account for the induction of discrete waves. Induction does not appear to depend on the counterion in the electrode because K^+ does not induce discrete waves when ejected from conventional KCl pipettes, and because Na^+ has been reported to desensitize *Limulus* receptors, but not to induce discrete waves (Lisman and Brown, 1972; Fein and Charlton, 1977*b*). A variety of anions have been injected into ventral photoreceptors in earlier studies, but they were not reported to induce the production of discrete waves. These anions include

ATP and GTP (Fein and Corson, 1981*b*), as well as Cl^-, SO_4^{2-}, and HEPES (Lisman and Brown, 1975). All of the pharmacological agents that induce discrete waves also bind calcium ions to some extent. However, ATP and GTP bind calcium, but do not induce discrete waves (Fein and Corson, 1981*b*). Furthermore, injection of the calcium chelator EGTA has not been reported to induce the production of discrete waves (Lisman and Brown, 1975).

Although the compounds that induce discrete waves are phosphatase inhibitors, it is unlikely that they exert their effects through a simple inhibition of metabolism, because known metabolic inhibitors (anoxia and dinitrophenol) do not induce the production of discrete waves, but instead cause a steady depolarization of the membrane and a decrease in the amplitude of the receptor potential (Lantz and Mauro, 1978). These effects are accompanied by an increase in the latency and time to peak of the response. The induction of discrete waves by the agents used in this study is not accompanied by an appreciable steady depolarization of the cell membrane or by desensitization and, as we will explain in the following article (Corson et al., 1983), the induction of discrete waves is not associated with an increase in the latency or time to peak of the response.

Distributions of Amplitudes of Discrete Waves

It is unlikely that the phosphatase inhibitors act by isomerizing the chromophore of rhodopsin because the chemically evoked waves are smaller, on average, than light-evoked waves. Although we were unable to resolve the peaks of the amplitude distributions for spontaneous and fluoride-induced discrete waves, it was clear that both kinds of waves are smaller than the light-induced discrete waves (see Fig. 6). Our observations of differences in the amplitude distributions of spontaneous and light-induced discrete waves are in agreement with differences reported by Behbehani and Srebro (1974) and Bayer and Barlow (1978) for discrete waves observed in voltage-clamped cells. They are also in accord with the original observation of these differences by Yeandle and Spiegler (1973) in unclamped cells. Thus, four separate groups have independently observed the same phenomena. In addition to the quantitative data for fluoride-induced discrete waves, we found by visual inspection that discrete waves induced by vanadate and GTP-γ-S (Fein and Corson, 1981*b*), as well as tungstate and molybdate, appear to be smaller than light-induced discrete waves. Yeandle and Spiegler (1973) have suggested that spontaneous waves might occur by thermal activation of a molecule other than rhodopsin. Similarly, we suggest that drug-induced discrete waves are smaller because the drugs may act at a molecule that follows rhodopsin in the pathway of transduction. It is possible that the molecular sites of origin of both spontaneous and drug-induced discrete waves are the same.

A Molecular Model of Phototransduction in Limulus

On the basis of observations reported here, we propose the molecular model of phototransduction shown in Fig. 6. Visual excitation is normally initiated

by photoactivation of rhodopsin (Rh → Rh*) in the cell membrane. At the molecular level, a membrane protein (P_{Na}) gives rise to the light-induced permeability increase underlying the light-induced current (I_L). The light-induced current in these cells is carried primarily, but not exclusively, by sodium ions (Millecchia and Mauro, 1969*b*). From the measurements of limiting conductance fluctuations by Wong (1978), it seems reasonable to assume further that the protein (P_{Na}) controlling permeability may be a gated pore with a conductance of ~18 pS and a mean open time of 18 ms. Recent results (Bacigalupo and Lisman, 1983) confirm the presence of a light-

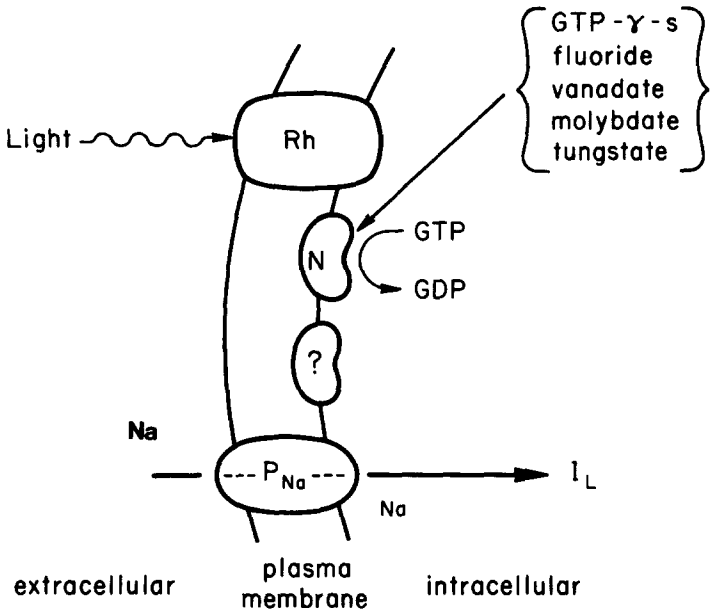

FIGURE 6. Proposed model for phototransduction in *Limulus* ventral photoreceptors. An incident photon activates a rhodopsin molecule (Rh), which interacts with a GTP-binding regulatory protein (N), which in turn activates other stages of amplification (designated "?"), finally resulting in an increase in the permeability (P_{Na}) of the cell membrane to sodium ions that are more concentrated outside the cell (larger "Na") than inside (smaller "Na"). Fluoride, vanadate, GTP-γ-S, molybdate, and tungstate are shown as inducing discrete waves by acting at an intracellular site, N. (See text for further details.)

activated pore in the cell membrane. On the basis of Wong's (1978) calculations, ~1,000 pores in a local area of several square micrometers are required to be open simultaneously during the production of a typical discrete wave. Because of the requirement for numerous pores to be activated by one photoactivated rhodopsin (Rh*), and because of the kinetic complexity of the waveform of the light response (Fuortes and Hodgkin, 1964), we assume that an enzymatic cascade is required to produce numerous messenger molecules that convey excitation from one Rh* to the surrounding pores during the characteristic time course of a discrete wave. For the sake of

simplicity, only one such pore is shown in Fig. 6. We now consider the implications our experiments have for other molecules that may be in this pathway.

Our findings suggest that the biochemical pathway of transduction can be activated by certain phosphatase inhibitors, and in particular by GTP-γ-S, at a step that is sufficiently close to the origin to produce large discrete waves and that precedes the step at which light adaptation occurs. However, it is unlikely that the phosphatase inhibitors act at rhodopsin itself because the waves are smaller than light-evoked waves.

We suggest that the site of drug action is a GTP-binding molecule (N, Fig. 6) that occurs early in the pathway of transduction. N would normally be activated by Rh*, and in its active form, N*, it would transmit excitation to subsequent molecules in the pathway. Activation and inactivation of N involves binding and hydrolysis of GTP. Normally, photoactivated rhodopsin promotes the binding of GTP to several N molecules. This event activates one or more additional enzymes (?, Fig. 6) whose reaction products control the gating of individual pores either directly or by means of subsequent stages. Hydrolysis of GTP to GDP at N would normally be one reaction that turns off the light response, although it is not likely to be the only such reaction because discrete waves clearly terminate in the presence of the drugs. Assuming a low rate of thermal activation of N, drug-induced discrete waves would occur by inhibition of the inactivation step (GTP hydrolysis). Similarly, we would predict that inhibition of the inactivation step would tend to prolong the light response, a prediction that we test in the companion paper (Corson et al., 1983).

In addition to the GTP-binding protein N, at least one other, unknown molecule (?, Fig. 6) is still required to account for the amplification inherent in drug-induced discrete waves, which, although they are smaller than light-induced waves, are still orders of magnitude larger than the single-channel events estimated by Wong (1978). Presumably, light adaptation affects these latter stages of transduction.

To date, the evidence for the hypothesis presented in Fig. 6 is the uniform action of the drugs used in this study plus the similar action of other GTP analogues (Bolsover and Brown, 1980). We have not yet been able to identify the biochemical site of action of the drugs. However, on the basis of our model, one would expect to find a light-activated GTPase in *Limulus* photoreceptors. Light-activated GTPase activity has been reported in the eyes of the octopus and the squid (Calhoon et al., 1980; Vandenberg and Montal, 1982), and recently a protein that may be a light-activated GTPase has been identified in the squid (Vandenberg and Montal, 1983).

Helpful Biochemical Analogies

In seeking the molecules that participate in visual transduction, we were guided in our early physiological experiments by an analogy with hormonal regulation of the enzyme adenylate cyclase. In the hormonal system, the application of the drugs used in this study leads to activation of adenylate

cyclase (Schwabe et al., 1979; Richards and Swislocki, 1979; Garcia and Haro, 1980), and therefore the drugs produce the hormonal response in the absence of the hormonal stimulus. The site of action for fluoride and GTP-γ-S is a GTP-binding regulatory subunit of adenylate cyclase (see Johnson et al., 1980). Recently, a similar GTP-binding protein has been isolated from vertebrate rods, where it is thought to regulate the activation of a phosphodiesterase by light (Fung and Stryer, 1980; see also Bitensky et al., 1981). As the analogy between the hormone-regulated adenylate cyclase system and the light-activated rod phosphodiesterase began to emerge, we were led to the attractive hypothesis that the drugs used in this study elicit single-photon responses in the absence of light in *Limulus* by activating a GTP-binding regulatory protein that is perhaps similar to the two mentioned above (Fein and Corson, 1981*b*). However, in the absence of biochemical confirmation of the presence and action of such a protein in *Limulus*, a number of unresolved questions prevent drawing the analogy between the model in Fig. 6 and the adenylate cyclase system on any but tentative terms.

The first question that arises from the hypothesis of an analogy between the hormonal system and the model of visual transduction in Fig. 6 concerns the activity of cholera toxin. Cholera toxin is a highly specific probe for GTP-binding proteins in hormonal systems (see Rodbell, 1980). It has been particularly useful there because it covalently labels such proteins by ADP ribosylation. Ribosylation inhibits the GTPase activity of the protein so that GTP causes quasi-irreversible activation of adenylate cyclase in a manner similar to the hydrolysis-resistant GTP analogues. Our initial expectation was that cholera toxin would excite ventral photoreceptors in a manner similar to the other pharmacological agents and provide a label for chemically identifying the protein. Our expectation rested on the assumptions that (*a*) a GTP-binding protein was present and involved in transduction, (*b*) it was sufficiently homologous to the other GTP-binding proteins for the toxin to recognize it, (*c*) the conditions for ribosylation by the toxin were present inside the cell, and (*d*) ribosylation by the toxin would inhibit the protein's GTPase activity and result in discrete-wave production. To date, we have been unsuccessful in demonstrating the induction of discrete waves as the result of the injection of activated cholera toxin (results not shown). A similar negative result has previously been reported by Brown et al. (1981). Presumably, the lack of effect reflects the failure of one or more of our initial assumptions in this line of investigation.

In addition to the lack of effect of cholera toxin, several uncertainties revolve around the relative activity of different nucleotides. Stern and Lisman (1982) found by using a dialyzed preparation that the rate of discrete waves became elevated when they left both ATP and GTP out of their internal dialysate for *Limulus* photoreceptors. Their findings do not necessarily indicate that the phosphatase inhibitors used in these studies act indirectly by lowering nucleotide levels. If anything, phosphatase inhibitors might be expected to elevate nucleotide levels by inhibiting their hydrolysis, and recent measurements of ATP concentrations indicate that ATP levels may in fact

increase in the presence of fluoride and vanadate (Rubin and Brown, 1983). Bolsover and Brown (1982) have also reported that ATP-γ-S, but not AMP-PNP (adenosine 2′,3′-imidotriphosphate), could stimulate discrete-wave production if followed by illumination. We have confirmed their findings, but at this point we cannot tell whether the effect of ATP-γ-S is due to a true affinity for ATP-γ-S at the site of discrete wave production or whether ATP-γ-S is converted to GTP-γ-S in the cell. Biochemical identification of the site of action of GTP-γ-S and the other phosphatase inhibitors in *Limulus* photoreceptors should resolve many of the ambiguities arising from experiments with cholera toxin and nucleotides.

Cyclic Nucleotides Are Unlikely to Be Involved in Excitation

Although a GTP-binding protein may be present in *Limulus* photoreceptors, and although all of the compounds used in this study activate hormone-regulated adenylate cyclase, it is unlikely that either cAMP or cGMP is involved in transduction in *Limulus* ventral photoreceptors. Excitation of *Limulus* photoreceptors by light is not accompanied by a rise in cAMP levels, as would be predicted if adenylate cyclase were involved (Schmidt and Farber, 1980). No change in cAMP levels in intact ventral photoreceptors accompanies excitation in 10 mM fluoride (Brown et al., 1981). No change in cGMP levels was found upon exposure of intact cells to illumination (Schmidt and Farber, 1980).

Direct tests of the action of cAMP and cGMP can be made by injection of the compounds or their analogues into ventral photoreceptors. If their synthesis is involved in excitation, one would expect to see either a smooth depolarization or possibly the induction of discrete waves on injection. If degradation is involved, the injection of excess substrate should desensitize the photoreceptors. In preliminary experiments, we have injected cAMP, cGMP, and the hydrolysis-resistant analogues cAMP-S and cGMP-S (Eckstein et al., 1974) into ventral photoreceptors. The results of these initial experiments did not appear to be compatible with a role for either cAMP or cGMP in excitation. These preliminary results are in accord with the report of Stern and Lisman (1982), who introduced cAMP and cGMP into ventral photoreceptors by the alternative technique of internal dialysis. If a GTP-binding protein is involved in excitation in *Limulus* photoreceptors, it seems likely that it may regulate enzymes other than adenylate cyclase or phosphodiesterase.

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