Enhancement and Phototransduction in the Ventral Eye of *Limulus*

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ABSTRACT *Limulus* ventral photoreceptors were voltage clamped to the resting (dark) potential and stimulated by a 20-ms test flash and a 1-s conditioning flash. At a constant level of adaptation, we measured the response to the test flash given in the dark (control) and the incremental response produced when the test flash occurred within the duration of the conditioning flash. The incremental response is defined as the response to the conditioning and test flashes minus the response to the conditioning flash given alone. When the test flash was presented within 100 ms after the onset of the conditioning flash we observed that: (*a*) for dim conditioning flashes the incremental response equalled the control response; (*b*) for intermediate intensity conditioning flashes the incremental response was greater than the control response (we refer to this as enhancement); (*c*) for high intensity conditioning flashes the incremental response nearly equalled the control response. Using 10-μm diam spots of illumination, we stimulated two spatially separate regions of one photoreceptor. When the test flash and the conditioning flash were presented to the same region, enhancement was present; but when the flashes were applied to separate regions, enhancement was nearly absent. This result indicates that enhancement is localized to the region of illumination. We discuss mechanisms that may account for enhancement.

INTRODUCTION

The present study arose from a chance observation on *Limulus* ventral photoreceptors. Cells were voltage clamped to the resting (dark) potential and stimulated by two flashes of light. Under certain conditions we observed that if the two flashes overlapped in time, the response (peak current) was greater than the sum of the responses to the two flashes each given separately. This phenomenon will be referred to as enhancement. We report here our observations of enhancement and discuss mechanisms that can account for enhancement. A brief account of these experiments has appeared previously (Fein and Charlton, 1976).

MATERIALS AND METHODS

The technique for preparing and the method of stimulating the ventral photoreceptors of *Limulus* have been described in previous papers (Fein and DeVoe, 1973; Fein and Charlton, 1975*a*,*b*). In this study the photoreceptors were impaled with two micropipettes, each mounted on a different micromanipulator. Before proceeding with voltage clamping, we established that the photoreceptor was isopotential by comparing the

photoresponses measured by the two electrodes. After determining that the photoreceptor was isopotential, we voltage clamped the cell to its resting (dark) potential. The voltage clamp was of conventional design. Clamp current was measured by a current to voltage converter. For all of the data presented in this paper the photoreceptor was continuously clamped to its resting potential and the photoresponse was measured as the light-induced membrane current. Throughout this paper we display inward membrane current as an upward deflection of the response. For all experiments the adequacy of the voltage clamp was monitored. The membrane potential never deviated more than 0.5 mV from the resting potential for the most intense stimuli used in these experiments. For dimmer stimuli the deviation of the membrane potential from its resting value was less than 0.5 mV, the deviation being proportional to the magnitude of the photocurrent generated by the light stimulus.

When a ventral photoreceptor is repeatedly stimulated with identical flashes of light, one observes that the response fluctuates in an apparently random manner (for example, see Fein and Lisman, 1975). These fluctuations are believed to be due to variations in the amplitude and number of the quantal events which summate to give the response (Fuortes and Yeandle, 1964; Dodge et al., 1968). These random fluctuations would tend to mask the phenomena we were trying to observe. Therefore, we used a Data General Nova 2 computer (Data General Corp., Southboro, Mass.) to average the responses to a number of stimuli. All of the data presented in this paper are computer averages (except where noted) of responses to repetitive stimuli.

Throughout this paper, light intensities (I) are given as $\log_{10} I/I_0$ where I_0 is the intensity of the unattenuated beam of white light which was used to stimulate the photoreceptors. The steady intensity of the light beam was calibrated at 520 nm (filter type G572-5200, Oriel Corp. of America, Stamford, Conn.) with a calibrated radiometer (United Detector Technology, Santa Monica, Calif., model no. UDT 111A). The calibrated photodiode was placed at the position normally occupied by the photoreceptor. The intensity of the white light was equated to 520 nm by using the voltage-clamped response of the receptor for comparison. The unattenuated beam of white light was found to be equivalent to 1.2×10^{15} 520 nm photons/cm^2-s. For uniform illumination of the photoreceptor (Figs. 1–6) the number of equivalent 520-nm photons incident on the photoreceptor for the unattenuated beam was calculated to be 6×10^{10}/s, if one assumes the size of the photoreceptor to be 50×100 μm (Clark et al., 1969; Stell and Ravitz, 1970). For three uniformly illuminated receptors we also measured the threshold for producing quantal events with light of 520 nm wavelength. The number of photons per second required to produce on the average one quantal event per second for the first receptor was 670, the second 530, and the third 510. This finding is in reasonable accord with that of Millecchia and Mauro (1969) who found that 10^3 photons per second produced on the average one quantal event per second. On the basis of this measurement with uniform illumination the 20-ms test flash we used throughout these experiments would produce on the average one quantal event if it contained the equivalent of between 510 and 670 photons of 520 nm wavelength. This result is in reasonable accord with that of Yeandle and Spiegler (1973) who found that from 452 to 952 photons of 540 nm wavelength are needed to produce one quantal event on the average. The threshold for producing one quantal event on the average with a 20-ms flash of white light corresponds to a log intensity of -6.25 to -6.35 in Figs. 1–6. In Figs. 7–9 the photoreceptor was illuminated with spots of light that were nominally 10 μm in diameter. Yeandle and Spiegler (1973) have shown that approximately the same number of photons are needed to produce a quantal event whether these photons are contained in a 10-μm spot or a large spot. Therefore, taking the ratio of the area of the cell to the area of the 10-μm spot to be about 65 to 1, we estimate that a lower

bound for the quantal event threshold in Figs. 7–9 corresponds to a log intensity of about −4.45 to −4.55.

Fig. 1 illustrates the experimental paradigm we used throughout this study. The photoreceptor was stimulated by two flashes of light, a 20-ms test flash labeled *T* and a 1-s conditioning flash labeled *S*. The stimuli were repeated every 10 s. 10 s allowed enough time for the computer to carry out all calculations between stimuli and minimized the time required to complete the necessary number of repetitions. Stimulus *S* was chosen to be much longer than *T* (chosen to be below the integration time of the photoreceptor) to insure that *S* would determine the adaptational state of the photoreceptor. In some cases, this precaution insured that the response to stimulus *S* was the same whether or not stimulus *T* preceded *S* (for example, see Fig. 1 B, *a* and *b* superimposed). More often when stimulus *T* preceded *S*, *T* would cause a small decrease in the peak of the response to *S* (for example, see Fig. 8 B, *a* and *b* superimposed; response *b* is greater than *a*). To insure that this small effect did not distort our measurements, we always compared the response obtained when *T* occurred during *S* (for example, see Fig. 8 A, *c*) to the response observed when *S* was given alone (for example, see Fig. 8 B, *b* and *c* superimposed). Throughout this paper we compare the response to stimulus *T* given in the dark (for example, see Fig. 1 C, $a - b$) to the incremental response produced when *T* occurs during the duration of *S* (for example, see Fig. 1 C, $c - b$). In order to keep the amount of data presented within reasonable limits (for example, see Fig. 3) we sometimes present only the response to *T* given in the dark (defined as I, see Figs. 1 and 2) and the incremental response produced when *T* occurs during *S* (defined as II, see Figs. 1 and 2). We refer to (I) as the control response and (II) as the incremental response. The delay time *t*, is defined in Figs. 1 and 2.

Because the photoreceptors were repeatedly stimulated every 10 s they never had enough time to fully dark adapt between stimuli. Therefore, all the results presented in this paper were obtained from partially light-adapted photoreceptors.

When measurements were made over many minutes, systematic drifts in the response of the photoreceptor would differentially affect the responses to stimuli given minutes apart. To eliminate this possible source of error, the different stimuli shown in Fig. 1 A *a*, 1 A *b*, and 1 A *c* were continuously interleaved in time. We used the computer to sort out the different stimuli and to keep a running average of the response to each stimulus.

RESULTS

Fig. 1 illustrates the typical results we obtained when we stimulated with relatively dim flashes. In Fig. 1 A we show the average response to each stimulus used. In Fig. 1 B we compare responses by showing them superimposed. And in Fig. 1 C we compare the control response to the incremental response. We invariably found, for dim stimuli, that the incremental response was essentially the same as the control response. That is, for dim stimuli, the light-induced currents appear to summate linearly.

When the intensity of stimulus *S* was raised 1.3 log units and *T* was raised 0.6 log units, the incremental response was larger than the control response. This result is presented in Fig. 2 C and was obtained from the same photoreceptor as in Fig. 1. This phenomenon, the enhancement of the incremental photoresponse over the control response, forms the basis of this study. To obtain a better understanding of this phenomenon, we systematically varied different parameters of the stimulus.

Fig. 3 is typical of the results obtained when the intensity of both T and S are kept constant and the delay time t is varied (see Figs. 1 and 2 for the definition of t). For small delay times (1, 20, 40, and 60 ms) the incremental response is biphasic and for longer delay times (80 and 100 ms) the incremental response is almost monophasic. This result seems to indicate that the negative component of the biphasic response is a phenomenon separate and distinct from enhancement. For t = 100 ms the negative component of the incremental response is absent yet enhancement is still present. Furthermore, Fig. 3 J shows that the responses for t = 80 and 100 ms have a duration that is nearly the same as the

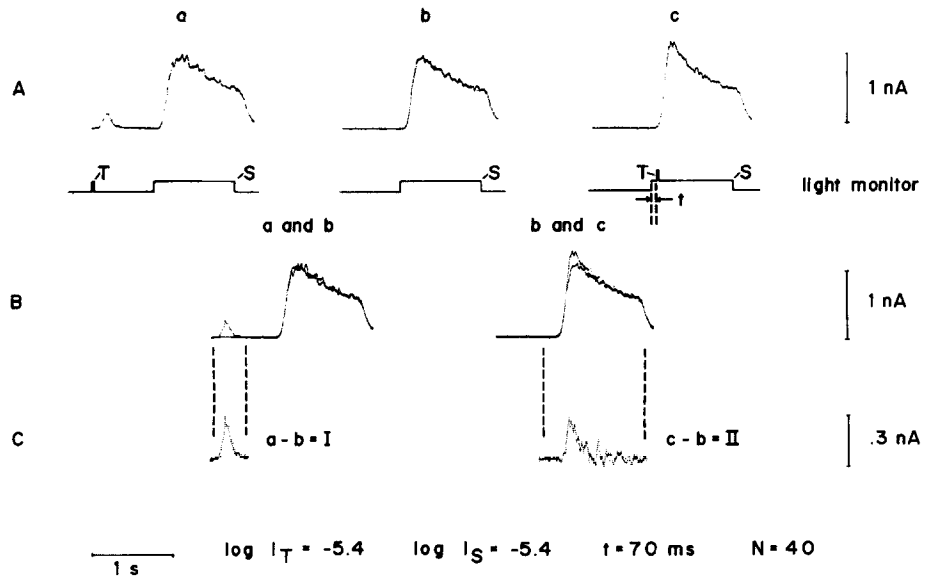

FIGURE 1. Linear summation of light-induced currents. In A, each trace is the average of N(40) responses to the stimulus shown by the light monitor. In B, tracings a and b of row A are superimposed, and tracings b and c of row A are superimposed. In C, the differences between a and b and between c and b are given. I_T is the intensity of stimulus T and I_S is the intensity of stimulus S. The threshold for producing on the average one quantal event corresponds to a log intensity of between −6.25 and −6.35 for stimulus T (see Materials and Methods).

duration of the positive component of the response for t = 1 ms. Also, in Fig. 3 G the responses of Figs. 3 A, B, and C are shown superimposed. Note that the postive components of the three responses are essentially identical whereas the negative component of the responses is not. This is shown more clearly in Fig. 3 H where it can be seen that the negative component of the incremental responses decreases systematically for longer delay times. We suspect that the negative component of the biphasic response is due to stimulus T adapting the photoreceptor and thereby reducing the response to stimulus S. This idea is borne out by our observation (not shown) that stimulus T causes a decrease in the peak of the response to stimulus S, when T precedes S (This point is more fully discussed in Materials and Methods). For these reasons (to be more fully

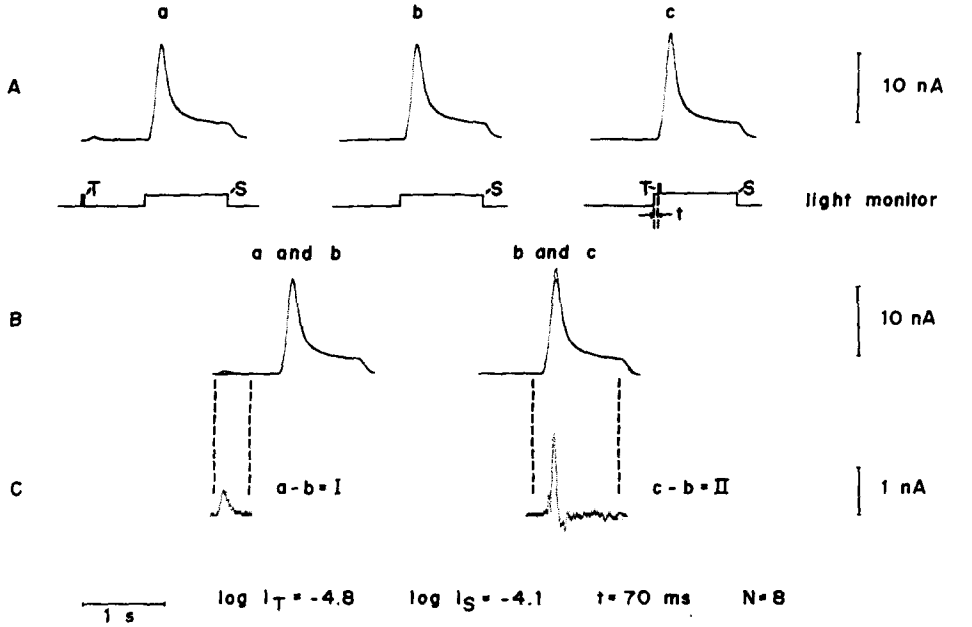

FIGURE 2. Enhancement of incremental response. Same cell as in Fig. 1. All symbols have the same meaning as in Fig. 1. The only difference between this figure and Fig. 1 is that I_T has been raised 0.6 log units and I_S has been raised 1.3 log units.

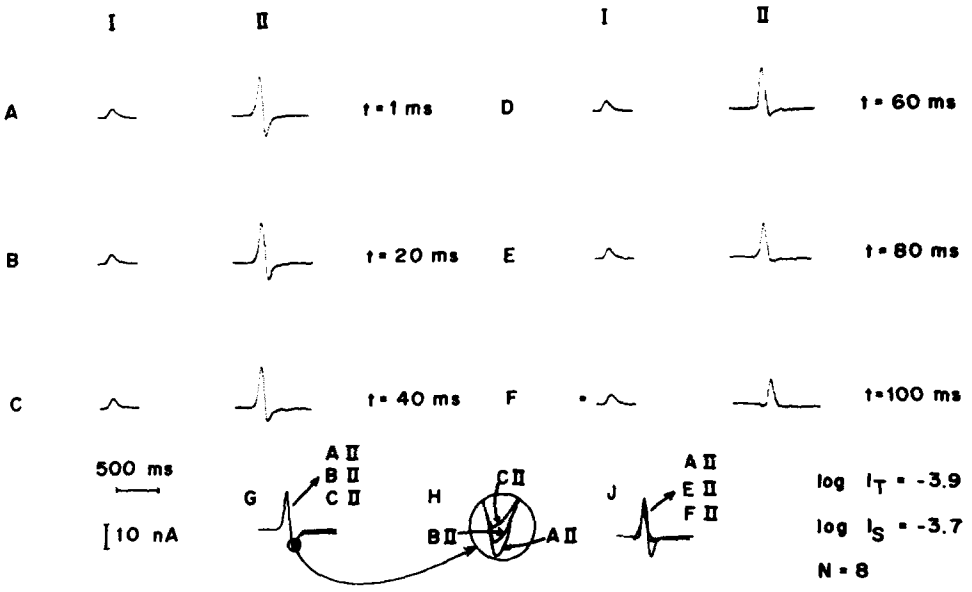

FIGURE 3. Effect of delay time t (see Figs. 1 and 2) on incremental response. Symbols I, II, I_T, I_S, N, and t are defined in Fig. 1. I_T, I_S and N are kept constant while t is varied from 1 to 100 ms. Only the control response (I) and the incremental response (II) are shown (see Materials and Methods).

analyzed in Discussion) we shall use the positive component of the incremental response as a measure of the incremental response. For delay times greater than 200 ms (not shown in Fig. 3) the incremental response was smaller than the control response. That is, enhancement appears to disappear beyond 200 ms of delay time. This apparent disappearance of enhancement is associated with the decrease in the size of the incremental response relative to the control response, that is with the onset of adaptation (Lisman and Brown, 1975).

Figs. 4 and 5 are typical of the results obtained when the intensity of stimulus T

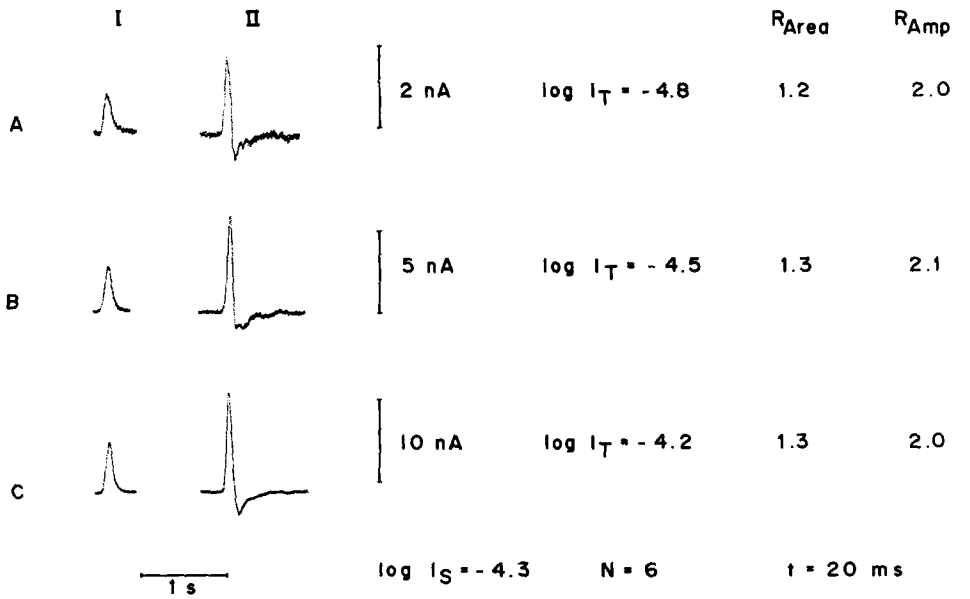

FIGURE 4. Effect of test intensity I_T on enhancement. Symbols I, II, I_T, I_S, N, and t are defined in Fig. 1. R_{area} is the area under the positive component of response II divided by the area of I. R_{amp} is the amplitude of the positive component of response II divided by the amplitude of I. The test intensity changes by a factor of 2 between A and B and between B and C. Note that the current scale changes by a factor of 2.5 between A and B and by a factor of 2 between B and C. Only the control response (I) and the incremental response (II) are shown (see Materials and Methods).

is varied while the delay time t and the intensity of stimulus S are kept constant. Fig. 4 shows typical results for a 20-ms and Fig. 5 for an 80-ms delay time. In both figures we use two indices of enhancement. We calculate the ratio of the amplitudes (R_{amp}, amplitude of positive component of response II divided by the amplitude of response I) and the ratio of the areas (R_{area}, area under positive component of II divided by area of I). Figs. 4 and 5 show that over the intensity range studied (0.6 log units) the enhancement is independent of the intensity of T (regardless of which index of enhancement is used). In other photoreceptors we have observed that this independence extends over a range of 1 log unit.

Fig. 6 is typical of the results obtained if the delay time is kept constant and the intensity of S and T are varied. It would be preferable to keep the intensity of T

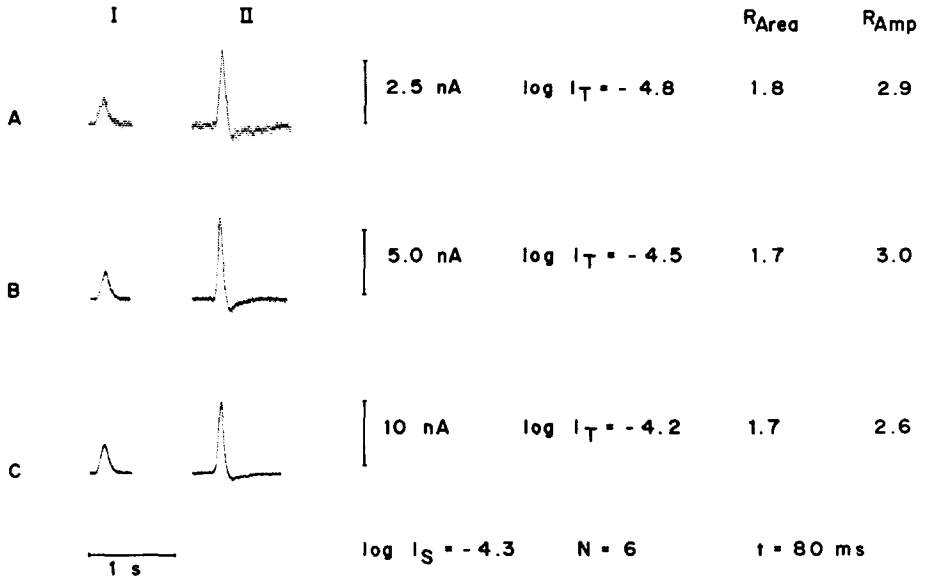

FIGURE 5. Effect of test intensity on enhancement. Symbols I, II, I_T, I_S, N, and t are defined in Fig. 1. R_{area} and R_{amp} are defined in Fig. 4. Different cell from that in Fig. 4. The test intensity changes by a factor of 2 between A and B and between B and C. The current scale changes by a factor of 2 between A and B and between B and C. Only the control response (I) and the incremental response (II) are shown (see Materials and Methods).

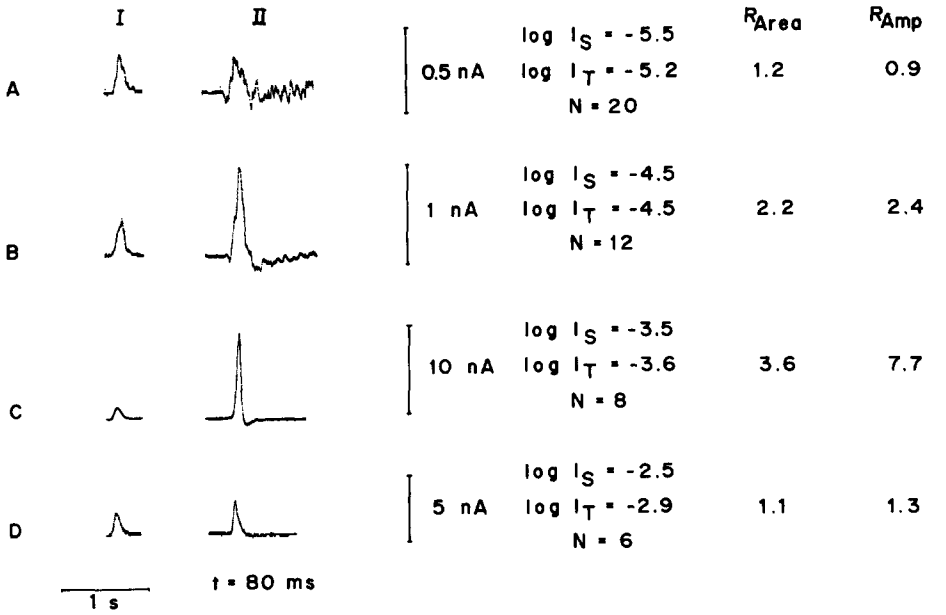

FIGURE 6. Effect of conditioning intensity I_S on enhancement. Symbols I, II, I_S, I_T, N, and t defined in Fig. 1. R_{area} and R_{amp} defined in Fig. 4. I_S changes by a factor of 10 between A and B, B and C, and between C and D. Only the control response (I) and the incremental response (II) are shown.

constant and only vary the intensity of S. This is not feasible because as we increase the intensity of S, the cell light adapts and the response to T decreases. Therefore, it is necessary to raise the intensity of T (so that we can measure the response to T) as the intensity of S is increased. As the intensity of S is increased we find that the degree of enhancement first increases and then decreases (independent of which index of enhancement is used).

We have previously shown that local illumination of part of a ventral photore-

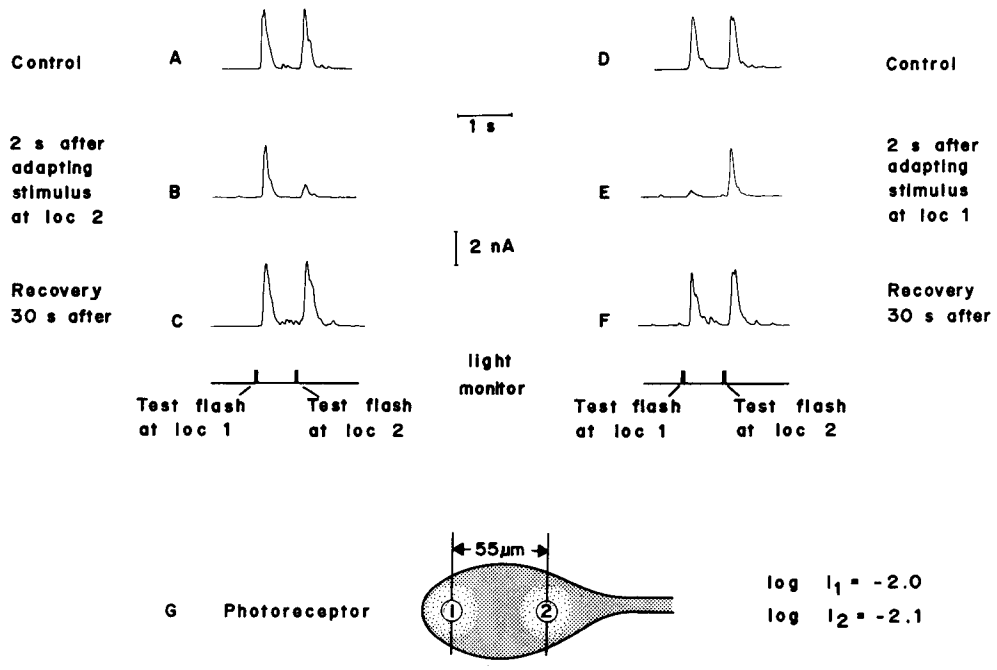

FIGURE 7. Localized desensitization produced by local adapting light in a voltage-clamped photoreceptor. G is a schematized version of the photoreceptor showing the two stimulus spots labeled 1 and 2. A–F show the light-induced currents elicited by two constant intensity 20-ms test flashes, one at location 1 and one at location 2. I_1 is the intensity of the test flash at location 1 and I_2 is the intensity of the test flash at location 2. The adapting stimulus had a duration of 8 s and had log intensity of −2.0 at location 1 and −2.1 at location 2. The responses shown are for single stimuli and are not computer averages.

ceptor leads to a localized flow of membrane current (Fein and Charlton, 1975*a*). Furthermore, it has been shown that the light adaptation produced by local illumination is localized to the region of illumination (Fein, 1973; Spiegler and Yeandle, 1974; Fein and Charlton, 1975*b*). Also, Fein and Lisman (1975) showed that injection of calcium ions into ventral photoreceptors locally desensitized the photoreceptor. These results led us to investigate whether enhancement would be localized to the region of illumination. Before we tested for whether enhancement was localized it was independently established that the separate regions of the photoreceptor we illuminated could be adapted locally, as was done by Spiegler and Yeandle (1974). Fig. 7 shows our control experiment

for this. Two spots of light, nominally 10 μm in diam, were focused onto regions 1 and 2 of the voltage-clamped photoreceptor (see Fig. 7 G for a schematic version of the stimulating situation). Fein and Charlton (1975*b*) give a detailed description of the photostimulator used in these experiments. An 8-s adapting stimulus at location 2 desensitizes the photoreceptor to a subsequent test flash at 2, whereas the response to a test flash at location 1 was nearly unaffected. A similar adapting stimulus at 1 desensitized the photoreceptor to a subsequent test flash at 1 while leaving the response to a test flash at 2 nearly unaffected. In both cases the photoreceptor recovered from the localized adapting stimuli in ~30 s. This result establishes that regions 1 and 2 of the cell can be adapted locally.

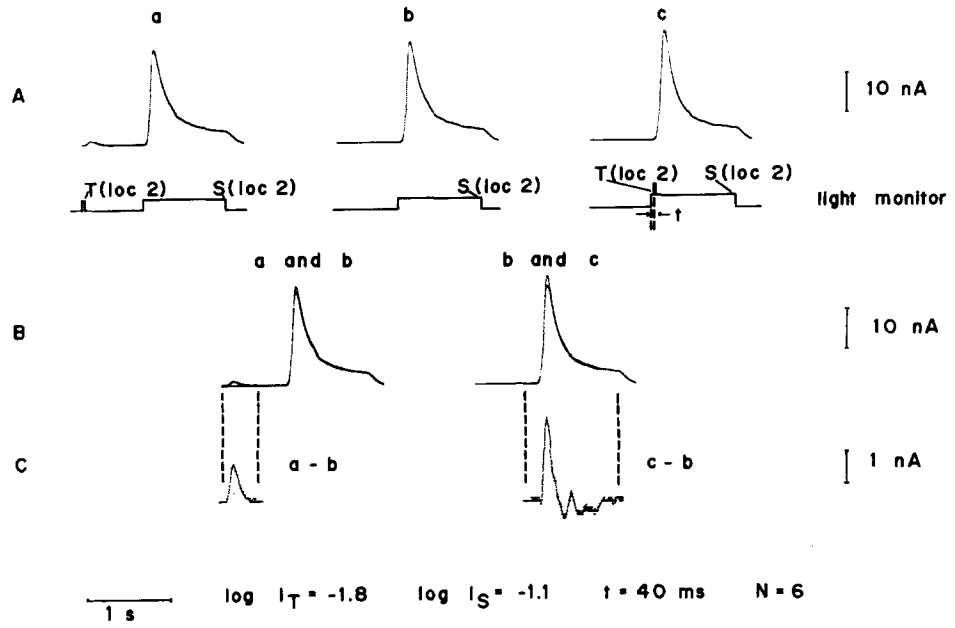

FIGURE 8. Enhancement of incremental response when stimuli are spatially superimposed. Symbols I_T, I_S, t, and N are the same as in Fig. 1. Same cell as in Fig. 7. Stimuli T and S are spots of light both focused at location 2 (see Fig. 7 G).

Next we set out to determine if enhancement is localized within these regions. Fig. 8 shows our test for enhancement when both spots of light were focused at location 2 (see Fig. 7 G) on the photoreceptor. One spot was used to flash stimulus T, the other stimulus S. Fig. 8 C shows that under these conditions enhancement is present. That is, when stimulus T and S are both flashed on the same region of the photoreceptor, enhancement is observed. Fig. 9 shows our test for enhancement when T and S are flashed on different regions of the same photoreceptor. The only thing that was changed between the experiments shown in Figs. 8 and 9 was the location of the spot of light that was used to deliver stimulus T. In Fig. 9 stimulus T was flashed on region 1 and stimulus S on region 2 (see Fig. 7 G). The data in Fig. 9 C indicate that enhancement is not present under these circumstances. Therefore, the results presented in Figs. 8

and 9 indicate that enhancement is localized to the region of illumination. We have also carried out this experiment under the condition where the intensity of stimulus T is adjusted to produce the same size control response at both positions 1 and 2. Under this condition we also find that enhancement is localized to the region of illumination.

DISCUSSION

A. Voltage-Clamped vs. Unclamped Photoreceptors

We have consistently observed enhancement in over 35 voltage-clamped photoreceptors. These findings clearly establish that, under the experimental proce-

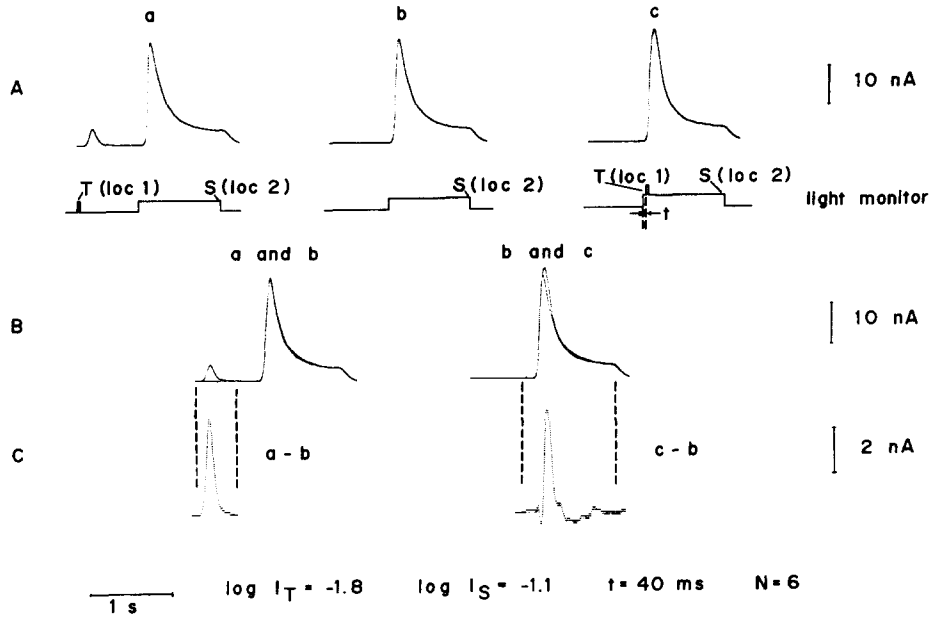

FIGURE 9. Linear summation of light-induced currents when stimuli are spatially separated. Symbols I_T, I_S, t, and N are the same as in Fig. 1. Same cell as in Figs. 7 and 8. Stimulus T is at location 1 and S at location 2 (see Fig. 7 G).

dures we use, enhancement is a property of these cells. Using the same program of light stimulation that produced enhancement in voltage-clamped receptors, we have searched for and failed to find enhancement in unclamped receptors where the photoresponse is a transmembrane depolarization (Millecchia and Mauro, 1969). We do not know why this is so, but perhaps the light-induced depolarization and decrease in input resistance (Fein and DeVoe, 1973) in the unclamped photoreceptor mask the enhancement of the incremental response. That is, for a given increment of conductance the voltage increment measured will depend on the input resistance and the net driving force for the ions involved. This might explain why enhancement has not been observed previously, since most studies on photoreceptors are not carried out under voltage clamp.

B. Enhancement and Adaptation

As mentioned in Results, we measured enhancement only if the delay time of the test flash was less than ~200 ms. For delay times greater than ~200 ms we measured adaptation; that is, the incremental response was smaller than the control response. We did not systematically measure the time when enhancement appears to change to adaptation; therefore, the 200-ms value should be considered only as an approximate estimate. Nevertheless, we can say that enhancement is observed only when the incremental response falls during the transient portion of the response to *S* (see Fig. 2 for example) and not during the steady state of the response to *S*. What we have not determined is exactly when, during the falling phase of the transient (in response to *S*, see Fig. 2), enhancement appears to change to adaptation. Lisman and Brown (1975) also carried out very similar experiments to these on ventral photoreceptors. They showed that the onset of adaptation took place during the falling phase of the transient of the photoresponse. On this point, our experiments confirm those of Lisman and Brown.

One might ask what is the time course of the onset of enhancement. Fig. 3 shows that we measured enhancement at delay times of 1 ms. This indicates that enhancement occurs with the onset of the response to stimulus *S*. Whether enhancement falls or is masked by adaptation cannot be answered by these experiments because adaptation occurs during the response to *S*. The apparent drop in enhancement (at longer delay times, see Fig. 3) might only be due to the onset of adaptation. The onset of adaptation might also explain why enhancement dropped in Fig. 6 D as the intensity of *S* was raised.

C. Quantification of Enhancement

In Results we gave a number of reasons for using the positive component of the biphasic incremental response as a measure of enhancement. We argued that the negative component of the biphasic response was due to stimulus *T* adapting the photoreceptor and thereby reducing the response to *S*. (This argument is similar to one given by Dodge et al., 1968, to explain why responses to incremental stimuli superimposed on steady backgrounds were biphasic.) If this is correct, we must explain why the negative component decreases as the delay time increases. The negative component of the incremental response occurs during the falling phase of the transient to *S* (see Fig. 2). Therefore, as the delay time is increased the negative component occurs closer to the steady state of the response to *S*. Lisman and Brown (1975) have shown that adaptation (due to *S*) occurs during the falling phase of the transient. Therefore, as the photoreceptor adapts to *S* (during the falling phase of the transient) the adapting effect of *T* will decrease as the delay time is increased. Even if the negative phase is subtracted from the positive phase in calculating the enhancement index, the results of Fig. 3 E and F and Fig. 6 clearly indicate that there is enhancement.

We measured enhancement as a function of the intensity of *S* (see Fig. 6) in four different photoreceptors. the maximum enhancement index (*R* area) varied by a factor of 3 between cells. We do not know what factors are responsible for this variation.

D. Conflicting Results

Before our work, Lisman and Brown (1972 and 1975) performed experiments similar to those reported here. They also sometimes measured an enhancement of the incremental response when the incremental response occurred near the peak of the transient. They assumed that the enhancement was due to an artifact of voltage clamping and pursued the matter no further. We feel that the data we have presented indicate that their assumption was incorrect and that the enhancement of the incremental response is not an artifact. Specifically, if the data in Figs. 4 and 5 were due to an artifact, the artifact would have to be perfectly graded with the intensity of stimulus T to produce the results of Figs. 4 and 5. Furthermore, the results of Fig. 6 are very difficult to reconcile with a voltage clamp artifact. If one assumed that the artifact increased as the clamp current increased and therefore as the intensity of stimulus S and T increased, this would explain Figs. 6 A, B, and C, but would not explain why the enhancement decreased in Fig. 6 D (for which we measured the largest current). Last and most important, a voltage clamp artifact could not explain the results of Figs. 8 and 9. If one assumed that an artifact was producing the results of Fig. 8, then one would expect to see a larger artifact in Fig. 9 (because the membrane currents in Fig. 9 are greater than in Fig. 8), yet there is no enhancement of the incremental response apparent in Fig. 9. For these reasons we feel that our results rule out the possibility that the enhancement is due to a voltage clamp artifact.

Lisman and Brown (1975) measured the peak light-induced current as a function of light intensity in dark-adapted photoreceptors. Sometimes they found that the current varied linearly with light intensity and sometimes they found that there was a region where the relationship was supralinear (the response-intensity curve plotted on log-log coordinates had a region where the slope was greater than one). They assumed that the supralinear relationship was due to a voltage clamp artifact. Since this supralinear relationship would also indicate enhancement we suggest that it is not an artifact, but rather represents another manifestation of enhancement. Our experimental results appear to be in accord with those of Lisman and Brown (1972, 1975); we disagree only with their interpretation.

Srebro and Behbehani (1974) also carried out experiments similar to these on ventral photoreceptors. These authors found results different from those we report here and those that Lisman and Brown (1972, 1975) have reported. Whereas we found either linearity (Fig. 1) or enhancement (Fig. 2) for small delay times, they found only adaptation. We can only say that we have never observed results similar to those they report, and apparently neither have Lisman and Brown (1972, 1975). This is very disturbing since all the experiments are done on the same preparation. We can offer no explanation for this discrepancy. However, we feel our measurements of localized enhancement (Figs. 8 and 9) suggest that our results are the correct ones, especially since this finding is consistent with our independent, nonvoltage clamp measurements of local membrane currents (Fein and Charlton, 1975*a*) and local adaptation (Fein and Charlton, 1975*b*).

Lisman and Brown (1975) found that at low light intensities the light-induced current was linear with light intensity. Our finding of linear summation of light-

induced currents (Figs. 1 and 6 A) is consistent with their finding. However, Srebro and Behbehani (1974) found significant response nonlinearity in ventral photoreceptors at low light intensities. Response linearity at low light intensities appears to be a property shared by many photoreceptors: squid (Hagins, 1965); rat rods (Penn and Hagins, 1972); turtle cones (Baylor and Hodgkin, 1973).

E. Localization of Enhancement

It has previously been shown that the adaptation of the receptor potential produced by illuminating part of a ventral photoreceptor tends to be localized to the region of illumination (Fein, 1973; Spiegler and Yeandle, 1974; Fein and Charlton, 1975*b*). The results presented in Fig. 7 extend these findings by showing that the adaptation of the light-induced current (measured under voltage clamp) produced by local illumination tends to be localized to the region of illumination. We have also shown that illumination of part of a ventral photoreceptor leads to a flow of local membrane current (Fein and Charlton, 1975*a*). Also, Fein and Lisman (1975) showed that injection of calcium ions into ventral photoreceptors locally desensitized the photoreceptor. Enhancement appears to be yet another aspect of the transduction process that is localized to the region of illumination (see Figs. 8 and 9).

It is intriguing to speculate that there is some cell structure that underlies the localization of these phenomena. First we will consider whether multiple photon absorptions by rhodopsin might account for enhancement. It has been found that between 450 and 1,000 photons are needed to produce on the average one quantal event (see Materials and Methods; Millecchia and Mauro, 1969; Yeandle and Spiegler, 1973). Assuming: (*a*) the photopigment in ventral eye cells of *Limulus* has a molar extinction similar to rhodopsin (40,600—Wald and Brown, 1953); (*b*) the quantum efficiency of isomerization is similar to other rhodopsins (0.65—Dartnall, 1972); (*c*) one quantal event is produced by the isomerization of a single visual pigment molecule (for examples see Fuortes and O'Bryan, 1972; Yeandle and Spiegler, 1973); (*d*) the size of the photoreceptor is 50 × 100 μm (Clark et al., 1969; Stell and Ravitz, 1970), we calculate, using Beer's law for dilute solutions, that there are between 4×10^8 and 9×10^8 visual pigment molecules in a ventral photoreceptor. These calculations are in reasonable accord with the prior findings of Lisman and Bering (1973) who estimated that ventral photoreceptors contain approximately 1×10^9 visual pigment molecules. Thus there would appear to be somewhere between 4×10^8 and 1×10^9 visual pigment molecules in a ventral photoreceptor. Assumption (*c*), together with our absolute calibration of the threshold for quantal events (see Materials and Methods), indicates that a 20-ms test flash of log intensity −6.3 isomerizes one rhodopsin molecule on the average. On the basis of this consideration we calculate that during the first 100 ms of stimulus *S* in Fig. 2 only 800 visual pigment molecules were isomerized. Reasoning similarly, we calculate that stimulus *T* in Fig. 2 isomerized fewer than 32 visual pigment molecules. When stimuli *S* and *T* were superimposed in Fig. 2 only 832 out of more than 4×10^8 visual pigment molecules were isomerized. Thus when enhancement was observed during the first 100 ms of *S* (Fig. 2) fewer than 1 in every 4×10^5 visual

pigment molecules were isomerized. The very small fraction of pigment molecules isomerized would appear to rule out the possibility that multiple photon absorptions by rhodopsin are a basis for enhancement. Also any reasonable variation of the four assumptions would not affect this conclusion.

Next we consider whether multiple photon absorptions within individual microvilli might account for enhancement. Langer and Thorell (1965) have directly shown in flies that the microvilli contain the visual pigment molecules. Therefore it is reasonable to assume that the microvilli seen in ventral photoreceptors (Clark et al., 1969) also contain the visual pigment molecules. There are no direct measurements of the number of microvilli in a ventral photoreceptor, therefore we have to estimate this quantity as follows. Taking the diameter of a microvillus as 0.07 μm and the length as 1 μm (Clark et al., 1969) gives a surface area of about 0.22 μm^2 for a microvillus. The number of rhodopsin molecules per microvillus can be calculated by assuming that *Limulus* rhodopsin is packed at the same density as frog rhodopsin. If a frog rod has 2×10^9 rhodopsin molecules (Hubbard, 1954) and the rod has a length of 50 μm and a diameter of 6 μm (Liebman and Entine, 1968) and a disk repeat distance of 300 Å (Korenbrot et al., 1973), we calculate that there are 2×10^4 rhodopsins/μm^2 in a frog disk. This gives 4.4×10^3 rhodopsins per microvillus. Using the estimates of the number of visual pigment molecules we calculate that there are between 9×10^4 and 2×10^5 microvilli per photoreceptor. We can also estimate the number of microvilli from the membrane capacitance. Millecchia and Mauro (1969) measured the membrane time constant for ventral photoreceptors and calculated a cell capacitance of between 0.004 and 0.010 μF. If one assumes a specific membrane capacitance of 1 $\mu F/cm^2$ (Cole, 1968) the calculated surface area for ventral photoreceptors is between 0.004 and 0.010 cm^2. Assuming that 90% of the surface area is made up of microvilli we calculate that there are between 1.6×10^6 and 4×10^6 microvilli per photoreceptor. Thus there would appear to be somewhere between 9×10^4 and 4×10^6 microvilli per photoreceptor.

We estimate that a photoisomerization occurs in less than 0.2% of the microvilli if we assume for the purposes of calculation that there are 9×10^4 microvilli and that the 160 isomerizations that are calculated to occur during the first 20 ms of stimulus *S* in Fig. 2 (see previous paragraph) occur within separate microvilli. If stimulus *T* (Fig. 2) isomerizes 32 visual pigment molecules as calculated and if a photoisomerization occurs in only 0.2% of the microvilli (during the first 20 ms of *S*) then we calculate, using the binomial probability law, that 6% of the time stimulus *T* will produce an isomerization in a microvillus in which stimulus *S* has produced an isomerization. This calculation assumed that stimulus *T* occurred with time delay $t = 0$ ms rather than the 70 ms shown in Fig. 2. This assumption allows the calculation to be made for the minimum number of isomerizations needed to produce enhancement. This assumption is justified by the data of Fig. 3. We chose our lowest estimate for the number of microvilli in making this calculation. If we had used our upper estimate for the number of microvilli, the calculated probability would be less than 0.2%. On the basis of the low value of these calculated probabilities (0.2–6%) it seems unlikely that enhancement is the result of multiple photon absorptions within a microvillus.

F. Enhancement and Quantitative Models of Phototransduction

The existence of enhancement puts strong constraints on models that might be proposed for the transduction process. For example, the models of Fuortes and Hodgkin (1964) for *Limulus* and Baylor et al. (1974) for turtle cones are linear models for the transduction process with delayed adaptation. These models, as formulated, do not account for enhancement.

G. Possible Mechanisms for Enhancement

Enhancement could be the result of cooperativity in the transduction process. Cooperativity is well known in the biochemical literature, for example, in the binding of oxygen to hemoglobin. Cooperative binding is usually determined by plotting the log of the ratio of ligand binding sites occupied to sites vacant vs. the log of the ligand concentration (Hill plot). If the Hill plot has a slope greater than 1 the binding is said to be cooperative. The hemoglobin molecule is a tetramer, containing four oxygen binding sites. The Hill plot for oxygen binding to hemoglobin has a region with slope greater than one. This has been interpreted to mean that binding oxygen to one site on the hemoglobin molecule *enhances* binding of oxygen to other sites on the same molecule. In the sense used in the biochemical literature *enhancement* of ligand binding would appear to be synonymous with *cooperativity* (see Van Holde, 1971, for a more detailed treatment of cooperativity).

In our experiments the photons in the light stimulus are analogous to the ligand and the photocurrent is analogous to the ligand binding. More than additive photocurrents (enhancement) are analogous to a slope greater than 1 on a Hill plot (see discussion of Lisman and Brown's [1975] work, second paragraph in part D). Thus our findings appear to be analogous with the term cooperativity as used in the biochemical literature. According to this analogy, enhancement might be produced if the rhodopsin molecules in ventral photoreceptors were organized into aggregates (probably greater than microvillus, see discussion part E) that cooperatively interact.

Cooperative interactions might also occur at some later steps in the transduction process beyond the visual pigment molecule. For example, suppose that the photoisomerization of rhodopsin brings about the production of a number of particles (Borsellino and Fuortes, 1968) which then interact with a "channel" (pore, carrier) to increase the permeability of the cell. The binding of the particles to the channels might produce enhancement. That is, the binding of the particles to one channel might facilitate the binding of particles to nearby channels. It could also be that the channels themselves interact. For example, the opening of one channel might facilitate the opening of nearby channels. These suggestions indicate that cooperativity could occur anywhere in the transduction process.

Cooperativity is not the only mechanism that could account for enhancement. For example, a great deal of indirect evidence indicates that there is an intermediate process interposed between the visual pigment molecules (rhodopsin) and the molecules which cause the permeability change that gives rise to the light-induced current (for example, see Fein and DeVoe, 1973; Fuortes and Hodgkin,

1964). As mentioned in the previous paragraph the photoisomerization of rhodopsin might bring about the production of a number of particles which interact with a channel (pore, carrier) to increase the permeability of the cell. Suppose that the process has a built-in safety factor; that is, more particles are produced per photoisomerized rhodopsin than are needed to open one channel. As the intensity of the stimulus is raised, the excess particles might accumulate and open some extra channels, thereby producing enhancement.

Any postulated mechanism for enhancement must account for two results described in this paper. First, enhancement is absent at low light intensities (Fig. 1), and second, enhancement is localized to the region of illumination (Figs. 8 and 9). In fact, the absence of enhancement at low light intensities is probably only a manifestation of the localization of the mechanism producing it. At low light intensities the photoisomerization of rhodopsins would be expected to be relatively farther apart compared to the higher light intensities where enhancement is observed. Since the photoisomerization of rhodopsin must initiate the events that lead to enhancement, at low light intensities the localized nature of the mechanism that produces enhancement would prevent enhancement from being observed.

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