Microvillar Components of Light Adaptation in Blowflies

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ABSTRACT The process of light adaptation in blowfly photoreceptors was analyzed using intracellular recording techniques and double and triple flash stimuli. Adapting flashes of increasing intensity caused a progressive reduction in the excitability of the photoreceptors, which became temporarily suppressed when 3×10^6 quanta were absorbed by the cell. This suppression was confirmed by subsequently applying an intense test flash that photoactivated a considerable fraction of the 10^8 visual pigment molecules in the cell. The period of temporary desensitization is referred to as the refractory period. The stimulus intensity to render the receptor cell refractory was found to be independent of the extracellular calcium concentration over a range of 10^{-4} and 10^{-2} M. During the refractory period (30–40 ms after the adapting flash) the cell appears to be "protected" against further light adaptation since light absorption during this period did not affect the recovery of the cell's excitability.

Calculations showed that the number of quantum absorptions necessary to induce receptor refractoriness is just sufficient to photoactivate every microvillus of the rhabdomere. This coincidence led to the hypothesis that the refractoriness of the receptor cells is due to the refractoriness of the individual microvilli.

The sensitivity of the receptor cells after relatively weak adapting flashes was reduced considerably more than could be accounted for by the microvilli becoming refractory. A quantitative analysis of these results suggests that a photoactivated microvillus induces a local adaptation over a relatively small area of the rhabdomere around it, which includes several tens of microvilli.

After light adaptation with an intense flash, photoactivation of every microvillus by the absorption of a few quanta produced only a small receptor response whereas photoactivation of every rhodopsin molecule in every microvillus produced the maximum response. The excitatory efficiency of the microvilli therefore increases with the number of quanta that are absorbed simultaneously.

INTRODUCTION

In invertebrates photoreceptor excitation takes place in highly specialized structures, the rhabdomeres, which are composed of a hexagonal array of microvilli (see El-Gammal et al., 1987). Receptor excitation is thought to be mediated by a bio-

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chemical amplification cascade triggered by light absorption through the visual pigment rhodopsin, which finally leads to the activation of ion channels (see Blumenfeld et al., 1986; Fein, 1986; Johnson et al., 1986; Paulsen and Bentrop, 1986; Devary et al., 1987). As a consequence of excitation the photoreceptors become light adapted, which was manifested by a reduction in the amplitude of the receptor response as well as by an acceleration of the time course of the response (Fuortes and Hodgkin, 1964; Dörrscheidt-Käfer, 1972; Laughlin and Hardie, 1978; Howard et al., 1987).

Two observations indicate that calcium plays an important role in the molecular events underlying light adaptation. Firstly, the prominent effects of light adaptation can be mimicked by raising the intra- or extracellular calcium concentration (Fulpius and Baumann, 1969; Millecchia and Mauro, 1969; Brown and Lisman, 1975; Fein and Charlton, 1978; Raggenbass, 1983; Hochstrate and Hamdorf, 1985; Brown, 1986). Secondly, a light-induced increase in the intra- as well as the extracellular calcium concentration actually occurs (Brown and Blinks, 1974; Brown et al., 1977; Maaz and Stieve, 1980; Levy and Fein, 1985; Minke and Tsacopoulos, 1986; Payne and Fein, 1987; Brown et al., 1988).

Light adaptation has also been found to occur locally, i.e., adaptation is most pronounced at the locus of prior illumination (Hamdorf, 1970; Fein and Charlton, 1975; Payne and Fein, 1983). This local adaptation may be explained by a local release of calcium, since it has been shown that the desensitization of the receptor caused by the injection of calcium is most pronounced near the locus of injection (Fein and Lisman, 1975).

These results, however, do not prove that light adaptation is exclusively mediated by calcium. The experiments presented in this article strongly suggest that other processes which are not dependent on a change in calcium concentration are involved in light adaptation. In particular, it is demonstrated that the photoreceptor cells become temporarily unexcitable after intense light stimulation, and that the stimulus intensity which is necessary to evoke this "period of refractoriness" is independent of the extracellular calcium concentration. It is argued that the refractoriness of the receptor cell is due to the refractoriness of the individual microvilli.

METHODS

Animal Material and Experimental Setup

Intracellular recordings of the electrical response of the receptors R1 through R6 to light were carried out on male white-eyed blowflies (*Calliphora erythrocephala* Meigen, chalky mutant). The larvae were reared on vitamin A rich liver in order to maximize the rhodopsin content in the rhabdomeres of the adult flies (Razmjoo and Hamdorf, 1976). Each microvillus in the rhabdomeres of such flies contains about 1,000 rhodopsin molecules (see Theory).

The preparation of the fly and the experimental setup were the same as described in Hochstrate and Hamdorf (1985). In short, the fly was mounted in a plastic holder and its head was hemisected horizontally close to the equator of the eye (cut preparation). The holder was fitted into a perfusion chamber in which the fly's head was steadily superfused with salines whose composition could be varied. At the beginning of each experiment the saline contained

130 mM NaCl, 0.1 mM $CaCl_2$, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid) pH 7.0 (standard medium). Higher calcium concentrations were obtained by the addition of appropriate amounts of $CaCl_2$ to the standard medium.

The perfusion chamber and the manipulator for the microelectrode were mounted on the object table of a microscope, by means of which the electrode was positioned in the receptor layer (under red light conditions) and the preparation was stimulated via the microscope objective. The orientation of the preparation was such that the receptor cells were illuminated perpendicular to their long axes, which guaranteed homogeneous light absorption over the entire length of the rhabdomere. The light stimulus was a 2 ms xenon flash of monochromatic green light (504 nm). A single flash led to a maximum absorption of 5×10^6 light quanta by the cell's rhodopsin, thereby photoactivating ~10% of the visual pigment in the rhodopsin state. However, after intense light adaptation higher absorption rates were found to be necessary to elicit a maximal receptor response. To achieve this the white flash was simply filtered through a pair of cut-off filters to exclude UV and IR radiation. A single white flash led to a maximum absorption of 2×10^8 light quanta per receptor, thereby photoactivating >95% of the cell's rhodopsin.

Determination of Light Absorption

Once a receptor cell had been impaled, the photoequilibrium between rhodopsin and metarhodopsin was adjusted to prevent changes in the rhodopsin concentration by the light flashes that were applied during the experiment. Depending on whether green or white flashes were used the preparation was illuminated with either continuous green light (504 nm) or intense flashes of white light from the xenon source. Since the wavelength of the green light is close to the isosbestic wavelength of the fly's rhodopsin/metarhodopsin system (Hamdorf and Schwemer, 1975), it leads to approximately equal amounts of visual pigment in the rhodopsin and metarhodopsin state. The rhodopsin content was higher when white light was used because of the higher absorbance of metarhodopsin (Schwemer, 1979). The relative rhodopsin content in the photoequilibrium established was determined photometrically to be 0.78.

After a dark period of 2 min the intensity-response function of the impaled cell was recorded in order to calibrate the efficiency of the xenon flashes. This method is based on the observation that the amplitude of the receptor response reaches half maximum when ~1,000 light quanta are absorbed. This number was extrapolated from low stimulus intensities at which the receptors respond with stochastically distributed quantum bumps (see Hamdorf and Kirschfeld, 1980*a*, *b*).

This relationship between the number of quantum absorptions and half saturation of the receptor response is in reasonable accord with the total quantum flux measured at the eye. Thus a flux of 2.5×10^{10} quanta (504 nm) per cm^2 during a 2-ms stimulus was found to evoke a half-maximum response. Considering the geometry and pigment content of the rhabdomeres (see Theory) it is estimated that this quantum flux leads to the absorption of 300 quanta by a rhabdomere illuminated perpendicularly to its long axis. The actual number is presumably somewhat higher due to light scattering in the preparation. These values agree with experimental data obtained from *Calliphora stygia* by Laughlin and Hardie (1978). They found that the receptor response was half saturated with a flux of 4.2×10^{10} quanta $cm^{-2} \cdot s^{-1}$ of monochromatic light with the most effective wavelength falling axially into the facet. Considering that the effective area of a facet is 500 μm^2 (Smakman et al., 1984) and the quantum capture efficiency of the photoreceptor is 0.5 (Dubs et al., 1981), we estimate that at half saturation the photoreceptor cell absorbs 500–1,000 quanta during the summation time of 7 ms (Hamdorf and Kaschef, 1965).

THEORY

Number of Microvilli and Rhodopsin Molecules in a Photoreceptor Cell

The number of microvilli and rhodopsin molecules within a receptor cell is of particular importance to the interpretation of the results presented below. Cross sections of fly ommatidia at the level of the nuclei revealed that the rhabdomere of the peripheral receptor cells (R1–R6) has ~20 microvilli over its width of 1 μm (see El-Gammal et al., 1987). Assuming the width of the rhabdomere is almost constant over its length (250 μm), we calculate that the rhabdomere of a receptor cell is composed of 10^5 microvilli.

The absorbance of the rhabdomeres of flies reared on a diet rich in vitamin A has been determined by microspectrophotometry to be 0.7 (Schwemer, 1979). This value corresponds to a rhodopsin concentration within the rhabdomeres of 0.7 mM (the molar extinction coefficient being $\epsilon_{max} = 4 \times 10^4$ liter · $M^{-1} \cdot cm^{-1}$; Stavenga and Schwemer, 1984). Taking the cross-sectional area of a rhabdomere to be 1 μm^2 (see Schwemer and Henning, 1984; El-Gammal et al., 1987), the number of rhodopsin molecules per microvillus is calculated to be 1.1×10^3. This corresponds closely to the value of 1.2×10^3 rhodopsin molecules per microvillus determined on the basis of electrophysiological measurements (Hamdorf and Razmjoo, 1979). From the density of particles in freeze-fracture preparations of rhabdomes (4,200 particles$\cdot \mu m^{-2}$, Schwemer and Henning, 1984), and allowing for the fact that this technique reveals maximally one half of the microvillus surface, we calculate that a microvillus contains at least 420 particles, most of which will be rhodopsin molecules.

Flies reared on a vitamin A–rich diet were used for the experiments presented in this article. From the above considerations it therefore follows that the total number of pigment molecules in these flies is about three orders of magnitude larger than the total number of microvilli.

Statistics of Quantum Absorption in the Microvillus Array

The average number λ_M of light quanta absorbed per microvillus was calculated by dividing the number of light quanta absorbed by the cell (Q_Σ) by the number of microvilli (N_Σ) in the cell's rhabdomere.

The fractions f_x of microvilli that absorb $x = 0, 1, 2, \ldots$ quanta are given by Poisson statistics:

$$f_x = \frac{e^{-\lambda_M} \cdot \lambda_M^x}{x!} \tag{1}$$

It follows from Eq. 1 that the fraction f_e of microvilli escaping photoactivation ($x = 0$) is:

$$f_e = e^{-\lambda_M} \tag{2}$$

and that the fraction $f_a = \Sigma_{x=1}^{\infty} f_x$ of light-activated microvilli is:

$$f_a = 1 - e^{-\lambda_M} \tag{3}$$

These equations can be applied if all microvilli have the same absorption probability, as is expected for a rhabdomere with a rectangular cross section. However, the cross section of the rhabdomeres in the fly is approximately semicircular (Fig. 1 *A*). Consequently, the probability of a microvillus absorbing a quantum of light is greater for the long microvilli in the center of the rhabdomere than it is for the short microvilli at the edges of the rhabdomere. The microvilli were therefore assigned to 10 classes according to their length (Fig. 1 *A*) and Poisson statistics were applied separately to each class.

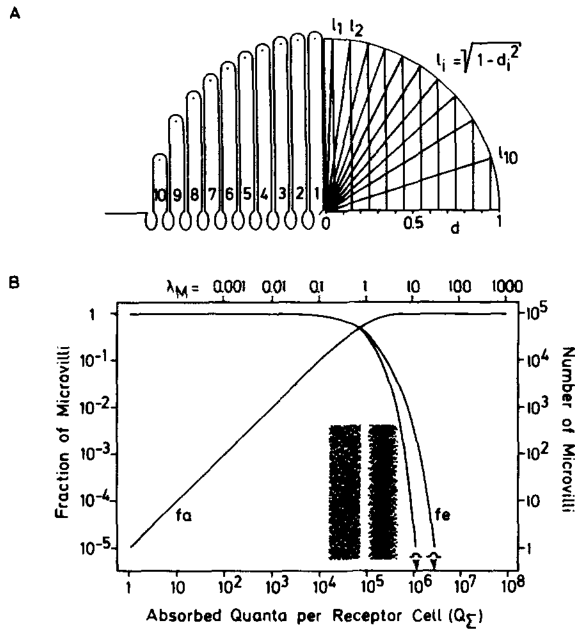

FIGURE 1. (*A*) Schematic drawing of a rhabdomere with a semicircular cross section used to determine absorption probabilities of microvilli. The relative length (l_i) of microvilli (10 length classes) was calculated according to Pythagoras from the relative distance *d* from the center of the rhabdomere. (*B*) Plots showing the fractions of microvilli that absorb light (f_a) or escape photoactivation (f_e) in relation to the number of light quanta absorbed by the receptor cell (Q_Σ). The fractions were calculated for a rhabdomere with a rectangular cross section (⎍, in which the microvilli have an equal probability of absorbing a light quantum) and a rhabdomere with a semicircular cross section (⌒, see *A*). The corresponding number of microvilli is given by the scale on the right-hand side. Due to the logarithmic scaling, both f_a functions coincide. Arrowheads mark the number of absorptions necessary to photoactivate every microvillus in the rhabdomere ($f_e < 10^{-5}$). The two inserts illustrate the pattern of photoactivated microvilli (black) for a rectangular (left) and a semicircular rhabdomere (right) after the absorption of $Q_\Sigma = 10^5$ light quanta by the cell ($\lambda_M = 1$)

The fraction $f_{a,i}$ of the microvilli in the *i*-th class, which absorb light, is given by:

$$f_{a,i} = 1 - e^{-Q_i/N_i} \tag{4}$$

where N_i is the number of microvilli in the *i*-th class and Q_i the number of light quanta absorbed. Q_i is proportional to the total number Q_Σ of quanta absorbed within the whole rhabdomere:

$$Q_i = P_i \cdot Q_\Sigma \tag{5}$$

where P_i represents the probability of quantum absorption. The value of P_i is proportional to the length of l_i of the microvilli. From $Q_\Sigma = \Sigma_{i=1}^{10} Q_i$ it follows that

$\Sigma_{i=1}^{10} P_i = 1$, which leads to:

$$P_i = \frac{l_i}{\sum_{i=1}^{10} l_i} \tag{6}$$

The calculated values of P_i varied between 0.146 for class 1 and 0.033 for class 10. From the number of photoactivated microvilli in the *i*-th class ($N_i \cdot f_{a,i}$), the total fraction of microvilli in the whole rhabdomere, which absorbs light, is calculated to be:

$$f_a = \frac{\sum_{i=1}^{10} N_i \cdot f_{a,i}}{N_\Sigma} \tag{7}$$

Since the 10 length classes contain equal numbers of microvilli ($N_i = N_\Sigma / 10$), Eq. 7 can be reduced to:

$$f_a = \frac{\sum_{i=1}^{10} f_{a,i}}{10} \tag{8}$$

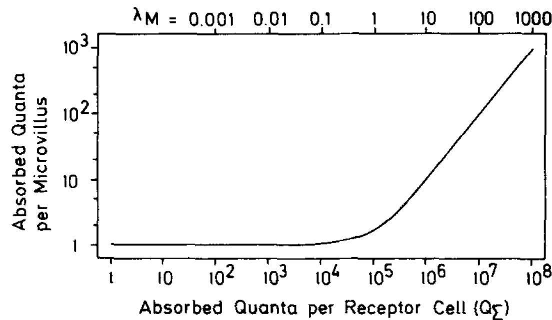

FIGURE 2. Average number of light quanta absorbed by the fraction f_a of photoactivated microvilli in relation to the number Q_Σ of quanta absorbed by the receptor cell.

Fig. 1 *B* shows a plot of the total fraction of microvilli which absorb light (f_a) and those which escape photoactivation ($f_e = 1 - f_a$) against the number of quanta absorbed during stimulation. The plot shows that f_a increases linearly with the number of absorbed quanta up to 10^4 quantum absorptions and approaches asymptotically the value 1 at higher intensities ($0.1 < \lambda_M < 10$). The fraction f_e is practically 1 at low quantum absorption rates but begins to drop sharply when more than 10^4 quanta are absorbed. Upon absorption of 2.8×10^6 quanta f_e is reduced to $<10^{-5}$, i.e., each of the 10^5 microvilli in the fly's rhabdomere becomes photoactivated. It has been noted that the plots of f_a and f_e calculated on the basis of a rectangular rhabdomere are similar to those for the semicircular rhabdomere, except that in the region $\lambda_M > 1$ fewer quanta are required to photoactivate a given fraction of microvilli. Thus the number of quantum absorptions necessary for the photoactivation of all microvilli, which is of particular importance for the interpretation of the results, amounts to 1.2×10^6, which is less than half of the number calculated for a semicircular rhabdomere (2.8×10^6).

The relationship between the average number of quanta absorbed per photoactivated microvillus ($Q_\Sigma/f_a \cdot N_\Sigma$) and the number of quanta absorbed by the cell (Q_Σ) is presented in Fig. 2. The plot shows that the microvilli predominantly absorb single light quanta when $Q_\Sigma < 3 \times 10^4$. The number of quantum absorptions per excited microvillus increases progressively when Q_Σ becomes larger than 3×10^4 and is proportional to Q_Σ when $Q_\Sigma > 3 \times 10^6$.

RESULTS

To investigate the different components contributing to the phenomenon of light adaptation, photoreceptor cells were stimulated by a sequence of two or three light flashes.

Receptor Responses to a Pair of Light Flashes at Low Calcium Concentration

The responses to light flashes recorded from receptor cells in the cut preparation were very similar to those obtained from cells in the intact eye when the preparation

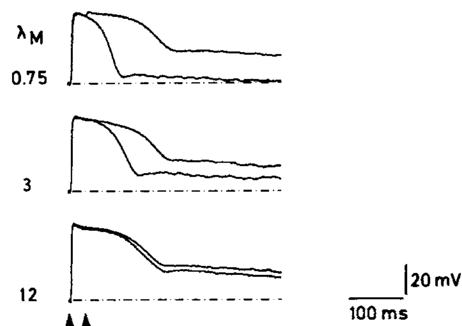

FIGURE 3. Response of a receptor cell to a pair of green (504 nm) flashes (an adapting flash followed 30 ms later by test flash) superimposed on the response to the adapting flash alone (lower trace in each case). The intensity of the adapting flashes is indicated by λ_M (the average number of light quanta absorbed per microvillus). That of the test flash was kept constant at $\lambda_M = 12$. Note that the response to the test flash was virtually abolished when the adapting flash led to the absorption of $\lambda_M = 12$ quanta per microvillus. The preparation was superfused with the standard medium containing a calcium concentration of 0.1 mM.

was superfused with the standard medium containing a low calcium concentration of 0.1 mM (Hochstrate and Hamdorf, 1985). A typical experiment with adapting flashes of varying intensity, followed 30 ms later by an intense test flash is presented in Fig. 3. The receptor response to the adapting flash alone became prolonged with increasing intensity ($\lambda_M = 0.75$, 3, 12) although its amplitude remained constant. With adapting flashes of low intensity ($\lambda_M = 0.75$) the test flash evoked a small additional depolarization and a distinct prolongation of the response. As the intensity of the adapting flash was increased the response to the test flash became progressively reduced and finally undetectable when $\lambda_M = 12$ light quanta were absorbed per microvillus (1.2×10^6 quanta absorbed by the cell). The slight difference between the response traces at $\lambda_M = 12$ was within the normal range of response variability.

Receptor Response to a Pair of Light Flashes at Higher Calcium Concentration

The question arises as to whether the excitation of the cell by the test flash is really abolished or only masked by the prolonged depolarization induced by the adapting flash. To answer this question, the same experiments were performed using a higher concentration of calcium in the superfusion medium. As shown earlier (Hochstrate and Hamdorf, 1985), when the extracellular concentration of calcium is increased the sensitivity of the receptors is reduced and the repolarization phase is markedly accelerated, particularly at high stimulus intensities. The latter effect would be expected to "unmask" the test response. At a calcium concentration of 1 mM the cell was still depolarized 30 ms after the adapting flash but it was almost completely repolarized when the calcium concentration was 10 mM (Fig. 4). Nevertheless, the response component due to the test flash, which was clearly detectable at $\lambda_M = 1.5$, was abolished when the intensity of the adapting flash was increased by a factor of 8 ($\lambda_M = 12$). This result shows that the intensity of the adapting flash that is necessary

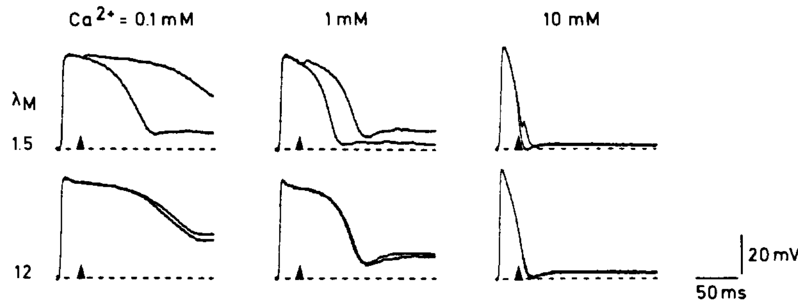

FIGURE 4. Responses of a receptor cell to a pair of green flashes superimposed on the response to the adapting flash alone (lower trace in each case) recorded at three different calcium concentrations (0.1, 1, and 10 mM). Otherwise the experimental conditions were the same as those for Fig. 3. Note that the test flash evoked a response at all three calcium concentrations when the adapting flash led to the absorption of $\lambda_M = 1.5$ quanta per microvillus. However, the response was effectively abolished when the intensity of the adapting flash was increased by a factor of 8 ($\lambda_M = 12$).

to suppress the test response hardly depends on the extracellular concentration of calcium.

Time Course of Light Adaptation

The time course of light adaptation was studied by varying the time interval between the adapting flash and the test flash (Figs. 5, 6, and 7). Fig. 5 shows the superimposed responses of a receptor cell to a pair of flashes, whereby the delay before the test flash ranged between 20 and 900 ms. The intensity of the adapting flash was increased stepwise by a factor of 2 ($\lambda_M = 0.25, 0.5 \ldots 64$), whereas that of the test flash ($\lambda_M = 64$) was kept constant throughout the experiment. The time interval of 30 s between recording each response trace was sufficient for the cell to regain the dark-adapted state, as can be deduced from the high reproducibility of the response to the various adapting flashes. At low-adapting intensities ($\lambda_M < 2$) every test flash

evoked a distinct response. However, with increasing adapting intensity the test response was temporarily suppressed. An average absorption of λ_M = 4–8 light quanta per microvillus was necessary to abolish the test response, which is in line with the data presented in Figs. 3, 4, and 9.

The diminution of the test response caused by the adapting flash depended on the delay of the test flash, being most prominent when the delay ranged between 30 and 40 ms. Within this range, an increase in the intensity of the adapting flash by a factor of only 8 was sufficient to diminish the amplitudes of the test responses from almost maximum (traces λ_M = 0.25) to ~0 (traces λ_M = 2). In contrast, to suppress the response to a test flash delayed by 80 ms to an equal degree, the intensity of the adapting flash had to be increased by a factor of at least 32 (compare traces λ_M = 1 and 32). Furthermore, the responses to test flashes delayed for longer than 100 ms were only moderately affected by the adapting flash.

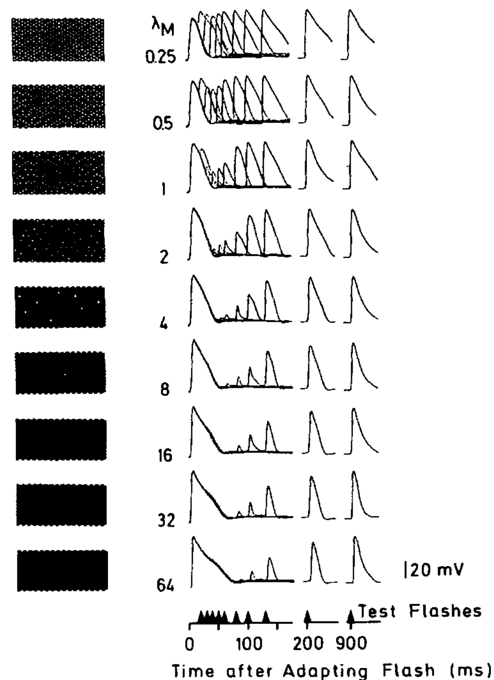

FIGURE 5. Responses of a receptor cell to pairs of green flashes. The initial adapting flash varied in intensity from λ_M = 0.25 to 64 and was followed by test flash after a delay of 20–900 ms. The intensity of the test flash was λ_M = 64. The 10 response traces recorded at each adapting intensity were superimposed. The time interval between recording each trace was 30 s. The drawings on the left-hand side illustrate the corresponding density of photoactivated microvilli for each intensity of the adapting flash. The calcium concentration in the superfusion medium was 10 mM.

The time course for the recovery of the test responses was little affected by the intensity of the adapting flash (Fig. 6). In particular, the initial slope of the recovery functions was approximately the same for all adapting intensities (λ_M > 2). However, the onset of recovery was progressively retarded as the intensity of the adapting flash was increased. The shift in the starting point of the recovery functions, from 30 to 70 ms, corresponds closely to the prolongation of the receptor response evoked by the adapting flash (see Fig. 5).

The effect of the adapting flash on the test response occurred over a period of ~30 ms. This can be seen in Fig. 5 by comparing the test responses that followed weak adapting flashes (λ_M = 0.25, 0.5, 1). The test responses evoked after a delay of

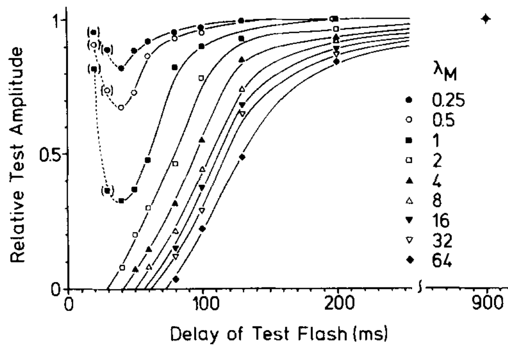

FIGURE 6. Data from the experiment shown in Fig. 5 showing the amplitude of the receptor response to the test flash (λ_M = 64) in relation to the time that it was delayed, plotted for each intensity of the adapting flash (λ_M = 0.25 to 64). Response amplitudes were measured with respect to the resting potential before stimulation and normalized to the amplitude of the response to the test flash delayed by 900 ms. Brackets indicate extensive overlapping of the response to the test flash and the adapting flashes.

20 ms are clearly larger in area than those after a delay of 30 or 40 ms; this is most evident for λ_M = 1. The time course of light adaptation is shown in more detail in Fig. 7. The receptor response to a pair of flashes applied with a delay of 5 ms between them (trace 2) was essentially the same as that evoked by synchronous flashes (trace 1), except that it was retarded by 5 ms in peak time and receptor repolarization, and a corresponding, further retardation was observed when the delay was 10 ms (trace 3). In contrast, with a delay of 15 ms a distinct acceleration of the repolarization process was observed (trace 4), i.e., the adapting effect starts with a delay of 10–15 ms. This range closely matches the time-to-peak of the response to the adapting flash alone (13 ms), leading to the conclusion that the start of light adaptation coincides with that of membrane repolarization. (This conclusion is in accordance with adaptation experiments performed under voltage-clamp conditions in *Limulus* ventral photoreceptors [Lisman and Brown, 1975].) The maximum effect of the adapting flash was observed using delay times of 30–35 ms, shortly before the

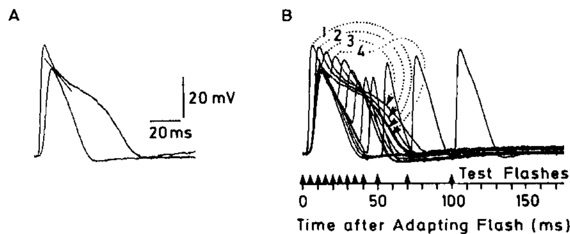

FIGURE 7. Development of the adapting effect of a green flash. (*A*) Superimposed receptor responses to the adapting flash (λ_M = 0.37) and to the test flash (λ_M = 23) applied separately. (*B*) Superimposed receptor responses to the adapting flash and the test flash applied after a delay time of 0, 5, 10, . . . , 35, 40, 50, 70, or 100 ms. The time interval between recording of the individual response traces was 40 s. The numbers *1–4* identify individual response peaks and their corresponding flanks.

cell was completely repolarized. The test resonse recovered when longer delay times were used (50–100 ms). The recovery of the amplitude occurred rapidly within a few hundred milliseconds but the complete recovery of the normal time course of the response took several seconds (not shown).

Adaptation as a Consequence of Receptor Excitation

Fig. 8 shows that 80 ms after an adapting flash (λ_M = 11.2, small arrowheads) the receptor cell could be excited again by a bright test flash (λ_M = 360, large arrowheads), as is also shown by Figs. 5 and 6. Surprisingly, the test response remained unchanged when an additional test flash was given 40 ms after the first (*B*), even though the additional flash alone was intense enough (λ_M = 11.2) to almost completely suppress the test response (*C*). Furthermore, the same result as in *B* was obtained when the intensity of the additional flash was greatly increased (λ_M = 180, *D*). The additional flash did not evoke a detectable receptor response in *B* or *D*. These results demonstrate that adaptation is closely linked to receptor excitation (*B*, *C*), but not to the amount of light-activated rhodopsin (compare *B* and *D*). This interpretation is further supported by a triple flash experiment presented in Fig. 9,

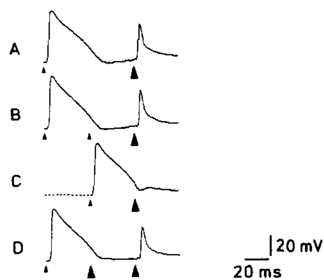

FIGURE 8. Suppression of light adaptation. (*A*) Receptor response to a pair of white flashes separated by a time interval of 80 ms. The initial adapting flash led to the absorption of λ_M = 11.2 light quanta per microvillus and the test flash to the absorption of λ_M = 360 quanta. (*B*) The response to the test flash was wholly unaffected by an additional flash (λ_M = 11.2) applied 40 ms after the adapting flash. (*C*) Effect of the additional flash on the response to the test flash when the adapting flash was omitted. (*D*) As in *B*, except that the intensity of the additional flash was increased by a factor of 16 (λ_M = 180). Note that this increase had no significant effect on the response to the last test flash.

which uses a series of stimuli similar to that used in the previous experiment (Fig. 8, *B* and *D*). In this case, the intensity of the first flash was varied over a wide range (λ_M = 0, 0.022 ... 11.2) while that of the second flash was kept constant at λ_M = 360, which was high enough to photoactivate ~50% of the visual pigment. With increasing intensity of the first flash, the response to the second flash was at first shortened at λ_M = 0.17, then its amplitude became reduced at λ_M = 0.7, and finally it was abolished at λ_M = 11.2. In contrast, the response to the third flash was affected in the opposite sense: a response was not evoked when the intensity of the first flash was low but it became clearly detectable at λ_M = 0.35 and increased further to a maximum at λ_M = 2.8. In other words, as the amplitude of the responses to the second flash was progressively suppressed, the response to the third flash became progressively more pronounced. This demonstrates that the effectiveness of the second flash in exciting the cell parallels its effectiveness in adapting the cell. The slight attenuation of the response to the third flash observed at λ_M = 5.6 and

11.2 is most probably due to the increase in the adapting effectiveness of the first flash (compare Figs. 5 and 6).

Correlation between Light Adaptation and Quantum Absorption in the Microvillus Array

The results presented so far demonstrate that after appropriate light adaptation fly photoreceptor cells become temporarily unexcitable. Light absorption during this refractory period neither evokes a receptor response (Figs. 3, 4, and 5) nor influences the state of adaptation (Figs. 8 and 9). The absorption of $\lambda_M = 10$ quanta per microvillus was necessary to suppress the response to a green test flash, which photoactivated up to 13% of cell's rhodopsin ($\lambda_M = 64$, Fig. 5). To abolish the receptor response to a white test flash of extreme intensity, which led to the photoactivation of most of the cell's rhodopsin, the intensity of the adapting stimulus had to be

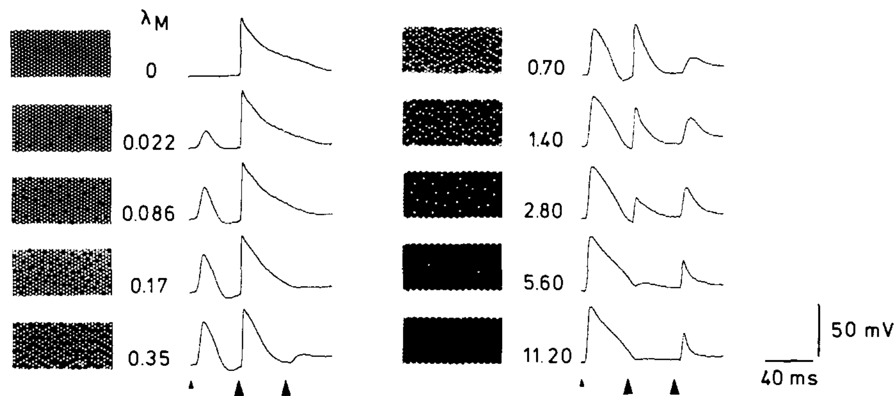

FIGURE 9. Receptor response to a sequence of three white flashes. The intensity of the first flash (*small arrowhead*) varied from $\lambda_M = 0$ to 11.2 as indicated. The intensity of the second and third flash was constant at $\lambda_M = 360$. The time interval between the flashes was 40 ms. Note that as the intensity of the initial adapting flash increases, the response to the second flash decreases, but that to the third flash increases. The drawings on the left of the traces illustrate the pattern of photoactivated microvilli generated by the initial flash.

increased to $\lambda_M = 30$ (see Figs. 10 and 12). This absorption value closely matches that necessary to photoactivate every microvillus ($\lambda_M = 28, f_e < 10^{-5}$; see Fig. 1). This coincidence favors the hypothesis that the refractoriness of the receptor is due to the refractoriness of the individual microvilli.

Receptor Sensitivity and Light Adaptation

In the experiments presented so far the effect of adapting flashes on the excitability of receptor cells as probed by intense test flashes has been analyzed. To determine the efficiency of the adapting flashes on the sensitivity of the photoreceptors, the intensity-amplitude functions of the cells were recorded. Recording was performed 40 ms after the adapting flash, i.e., at the moment of maximum adapting effect. The result (Fig. 10) shows that as the intensity of the adapting flash is increased, the

intensity-amplitude function is progressively shifted to higher stimulus intensities, e.g., after an adapting stimulus that led to the absorption of 4.6×10^4 light quanta, the intensity-amplitude function was shifted by 2.5 $\log_{10}$ units, corresponding to a loss in sensitivity by a factor of 300. Increasing the intensity of the adapting flash above 4.6×10^4 absorbed quanta caused a further shift of the intensity-amplitude function and a parallel reduction of the maximum response. Note that within the intensity range of 9.2×10^4 to 3×10^6 absorbed quanta a maximum response was only evoked by a bright test flash (up to 2×10^8 absorbed quanta per cell), which photoactivated most of the cell's rhodopsin. No response was detected when the cell

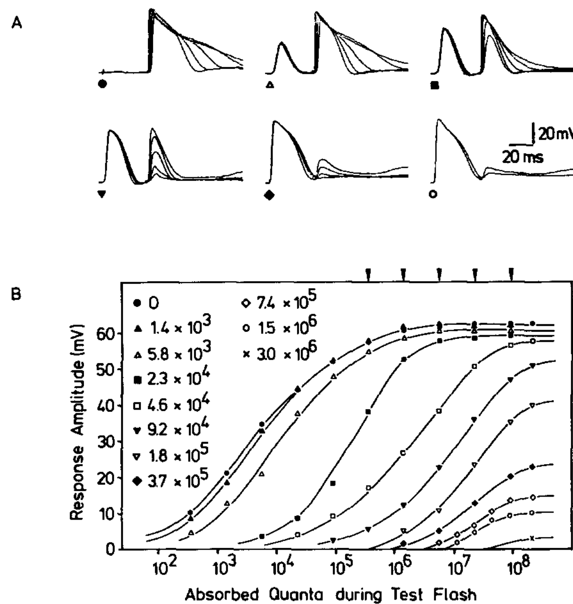

FIGURE 10. Effect of light adaptation on the intensity-amplitude function. (*A*) Superimposed receptor responses to a pair of flashes separated by an interval of 40 ms using six different intensities of adapting flash (as indicated by the corresponding symbols in *B*). The intensity of the test flashes is marked in *B* by the arrowheads. At the two highest intensities (◆, ○) only the more intense test flashes were applied. (*B*) Intensity-amplitude functions measured 40 ms after adapting flashes of different intensities (listed on the left). The functions were obtained by plotting the amplitude of the test response evoked after an adapting flash of equal intensity vs. the number of quanta absorbed during the test flash. The symbols indicate the number of light quanta absorbed by the cell during the adapting flash.

absorbed more than 3×10^6 light quanta during the adapting flash ($\lambda_M > 30$, see Fig. 12).

A plot of the relative sensitivity of the receptor cells vs. the number of light quanta absorbed during the adapting flash (Fig. 11) shows that the sensitivity was unaffected when less than ~1,000 light quanta were absorbed during the adapting stimulus. After the absorption of 10^4 quanta, however, the sensitivity dropped to 0.1 and was further reduced to 10^{-5} when the adapting flash led to the absorption of 10^6 quanta. The absorption of $>3 \times 10^6$ light quanta reduced the sensitivity to zero, as indicated by the fact that even light stimuli which photoactivated >90% of the

cell's rhodopsin were unable to evoke a detectable response. In contrast to receptor sensitivity, the maximum response remained almost unaffected up to 10^5 quantum absorptions, but then it sharply dropped and became undetectable when $>3 \times 10^6$ quanta were absorbed.

DISCUSSION

Photoreceptor Refractoriness as a Consequence of Microvillus Refractoriness

The experiments presented in this article demonstrate that after an intense adapting flash the photoreceptor cells of blowflies become temporarily unexcitable as probed by test flashes of extreme intensity. This refractoriness of the receptor cell occurred if the cell had absorbed $\sim 3 \times 10^6$ light quanta during the adapting flash ($\lambda_M = 30$). This absorption number is markedly lower than that necessary to photoactivate the bulk of the cell's visual pigment in the rhodopsin state (Fig. 11). Therefore, the possibility that receptor refractoriness is due to the depletion of excitable visual pigment can be excluded. However, the absorption number necessary to evoke receptor refractoriness closely matches the number of quantum absorptions necessary to photoactivate every microvillus in the fly's rhabdomere ($\lambda_M = 28$, see Theory). This coincidence favors the hypothesis that the refractoriness of the receptor cell is due to the refractoriness of the individual microvilli.

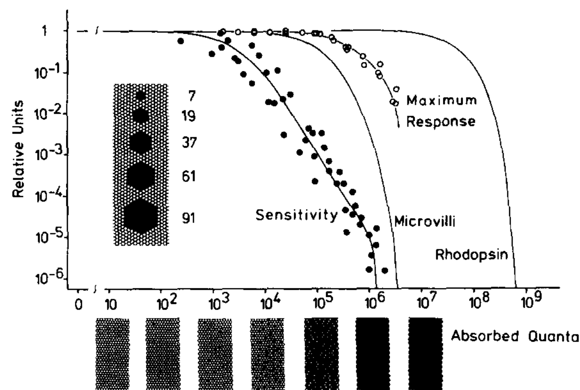

FIGURE 11. Relative receptor sensitivity and maximum response amplitude in relation to the number of light quanta absorbed by the cell during the adapting flash. Relative sensitivity was defined as the reciprocal of the factor by which the stimulus intensity had to be increased in order to evoke the same criterion response (10 mV) in the light-adapted state as in the dark-adapted state. The sensitivity data (*closed circles*) were obtained from experiments on six different cells. Three experiments were performed with green flashes and three with white flashes. The maximum receptor response data (*open circles*) were taken from the three experiments using white flashes. For comparison, the fraction of microvilli and of rhodopsin molecules escaping photoactivation by the adapting flash are also plotted (the former for a semicircular rhabdomere; see Theory). The inset illustrates the relative size of the area of locally adapted microvilli (7, 19, 37, 61, and 91 microvilli) when the microvillus in the center of the area is photoactivated (see Discussion). The drawings at the bottom of the figure illustrate the pattern of photoactivated microvilli generated by the absorption of 10–10^7 light quanta during the adapting flash.

Accordingly, after a weak adapting flash the response to the test flash should be produced by those microvilli that previously escaped photoactivation by the adap-

ting flash. The fraction of microvilli escaping photoactivation (f_e) decreases sharply over a narrow range of intensity (Fig. 1), and therefore, the amplitude of the test response should drop from maximum values to zero within this intensity range. This, however, does not mean that the relative amplitude of the test response is simply given by the fraction of microvilli that escaped photoactivation (see Figs. 11 and 12). Rather, the relationship between microvillus photoactivation and the response to test flashes of extreme intensity was found to be similar to that in dark-adapted cells (Fig. 12). For example, after the absorption of 2×10^5 quanta during the adapting flash the fraction of microvilli escaping photoactivation is $f_e = 0.2$. Provided that only this fraction contributes to the receptor response elicited by the test flash, the test response should be due to the photoactivation of 2×10^4 microvilli (total number of microvilli $N_\Sigma = 10^5$). As seen in Fig. 12 the relative amplitude of the test response was 0.7, and almost the same amplitude was elicited by the photoactivation of 2×10^4 microvilli in dark-adapted cells (see also Fig. 10). This

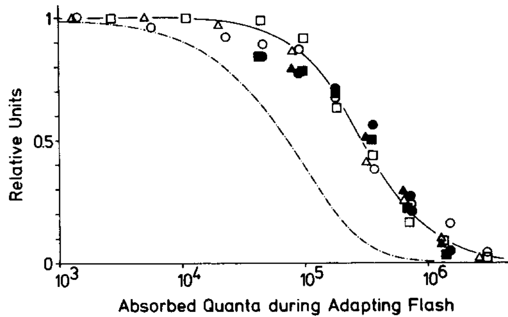

FIGURE 12. Semilogarithmic plot of the maximum response amplitude recorded after light adaptation vs. the number of light quanta absorbed during the adapting flash (*open symbols*, data from Fig. 11). The broken curve shows the fraction of microvilli escaping photoactivation by the adapting flash (f_e) calculated on the basis of a semicircular rhabdomere (see Theory). In addition the closed symbols show the response amplitudes elicited in dark-adapted cells by light stimuli that photoactivated the same number of microvilli as escaped photoactivation by the different adapting flashes.

result shows that photoactivation of equal numbers of microvilli leads to responses of approximately equal amplitudes irrespective of whether the cells are dark or light adapted. It is emphasized that this equivalence is only valid if in the light-adapted state the responses were elicited by test flashes of extreme intensity (see below).

The equivalence of the response amplitudes in this particular case allows us to estimate the minimum number of microvilli that are necessary to induce a just detectable test response in the light adapted state. At the Ca concentration of 10 mM used in the experiments, the absorption of ~10 quanta evoked a just detectable response in dark-adapted cells (see Hochstrate and Hamdorf, 1985). Correspondingly, in light-adapted cells the test response is expected to become undetectable, if <10 microvilli escaped photoactivation, i.e., if f_e becomes $<10^{-4}$. The number of quantum absorptions necessary to achieve this is calculated to 2.1×10^6 ($\lambda_M = 21$). This value is only slightly smaller than that necessary to photoactivate all microvilli in

the fly's rhabdomere (λ_M = 28) and still in reasonable accordance with the experimental results (λ_M = 30).

The above considerations imply that a single quantum absorption is sufficient to render a microvillus refractory. This view is supported by the result of the triple flash experiments discussed in the following section.

Protection against Light Adaptation Is Caused by Microvillus Refractoriness

The receptor cell in the refractory state appears to be "protected" against further light adaptation, since the regeneration of receptor excitability was not appreciably affected by light absorption during the refractory period (Fig. 8, compare traces *A* and *D*). Like the refractoriness of the receptor cell, this protection against light adaptation also seems to be caused by the refractoriness of the individual microvilli, as deduced from the result of the triple flash experiment presented in Fig. 9. In this experiment a response to the third flash could be clearly detected when ~30% of the microvilli (λ_M = 0.35) were excited by the first flash, and this third response became maximum when the first flash had excited the main fraction of the microvilli (94% if λ_M = 2.8). Thus the modulation of the receptor response to the third flash occurred exactly when substantial fractions of microvilli were excited by the first flash and thereby became resistant to light adaptation by the second flash. This resistance could be induced by rather weak stimuli (λ_M = 0.35), which predominantly led to the absorption of only one light quantum per excited microvillus (see Fig. 2). Therefore, the absorption of a single light quantum seems to be sufficient not only to make the microvillus refractory but also to protect it against further light adaptation.

Since many aspects of light adaptation can be mimicked by raising calcium inside and/or outside the cell (Brown and Lisman, 1975; Raggenbass, 1983; Hochstrate and Hamdorf, 1985), it may be that calcium also mediates the refractoriness of the microvilli. However, this seems improbable since approximately the same intensity of adapting flash is necessary to suppress the excitability of the cell at low and high calcium concentrations (Fig. 4). At the very least, this finding demonstrates that microvillus refractoriness cannot be caused by a light-induced influx of calcium (see Ivens and Stieve, 1984).

Local Adaptation in the Microvillus Array

Microvillus refractoriness should lead to a specific reduction in receptor sensitivity. The sensitivity relative to the dark-adapted state should be simply given by the fraction of microvilli escaping photoactivation by the adapting stimulus, provided that the excitatory efficiency of these microvilli is the same as in the dark-adapted state. However, when $>10^3$ quanta were absorbed during the adapting flash the loss in sensitivity was found to be much larger (Figs. 10 and 11), which indicates that the excitatory efficiency of the microvilli is reduced. The reduction is given by the discrepancy between the experimental sensitivity data and the fraction of microvilli escaping photoactivation (Fig. 11).

It is likely that the excitatory efficiency of the microvilli that surround a photoactivated microvillus is predominantly reduced. This local adaptation could well be

caused by a desensitizing factor that spreads from the photoactivated microvillus. The obvious candidate for this factor is calcium, stemming either from a local influx through the light-activated ion channels or from a local release from intracellular stores (Fein and Lisman, 1975; Fein and Charlton, 1975; Brown et al., 1977; Ivens and Stieve, 1984; Payne and Fein, 1987).

A single photoactivated microvillus is estimated to affect (locally adapt) maximally 100 microvilli since receptor sensitivity was essentially unchanged when <1 in 100 microvilli had absorbed light during adaptation ($f_e/f_a \approx 100$; corresponding to 10^3 quantum absorptions). On the other hand, photoactivation of 1 in 30 microvilli (3×10^3 quantum absorptions) reduced the relative sensitivity to ~0.3. This loss is only explicable when at least 20 microvilli were locally adapted, even under the extreme assumption that the microvilli were completely desensitized. Therefore, it is concluded that a single photoactivated microvillus reduces the excitatory efficiency of several tens of microvilli in its surroundings. When more than 3×10^3 quanta were absorbed during the adapting flash the excitatory efficiency of the microvilli progressively decreased as indicated by the growing discrepancy between the sensitivity data and the calculated fraction of microvilli escaping photoactivation (see Fig. 11). This effect is thought to be due to the progressive overlapping of the areas of locally adapted microvilli. The maximum effect appears to occur when every microvillus that escaped photoactivation is effectively surrounded by refractory microvilli (that is, between $\lambda_M = 1$ and $\lambda_M = 2$; compare graphs in Figs. 5 and 9). Thus, after the absorption of 2×10^5 light quanta ($\lambda_M = 2$) the receptor sensitivity was ~500 times less than expected based on the microvillus refractoriness alone (relative sensitivity = 3×10^{-4}, $f_e = 0.17$). The further drop in sensitivity observed after more intense adaptation appears to be directly correlated with the decrease in the fraction of microvilli that escaped photoactivation.

The Excitatory Efficiency of the Microvilli Depends on the Number of Absorbed Quanta

The excitatory efficiency of a microvillus depends not only on the effects of local adaptation by neighboring microvilli but also on the number of light quanta the microvillus absorbs. In the dark-adapted state the amplitude of the receptor response was saturated after every microvillus had been photoactivated (3×10^6 quanta absorbed by the cell, Fig. 10 *B*), and multiple quantum absorptions by the individual microvilli are reflected in the prolongation of the receptor response (Fig. 10 *A*). In the light-adapted state, the amplitude of the test response also depends on the number of quanta each excitable microvillus absorbs, as can be deduced from the intensity-amplitude functions shown in Fig. 10 *B*. Consider, for example, the intensity-amplitude function recorded after adaptation with 9.2×10^4 quanta. A distinct response was first obtained when ~10^5 quanta were absorbed by the cell and the response began to saturate when >10^8 quanta were absorbed. In the lower part of the curve, up to 2.8×10^6 absorbed quanta, the fraction of photoactivated microvilli increased only from 0.63 to 1. When more than 2.8×10^6 quanta were absorbed the test flash photoactivated every microvillus so that only the increasing number of quantum absorptions per microvillus can be responsible for the further increase in response amplitude (see Fig. 2). This result indicates that the effective-

ness of the transduction cascade within a microvillus increases in proportion to the number of light-activated rhodopsin molecules.

The "excessive" production of messenger molecules as a result of multiple quantum absorption may well cancel the effect of the putative adapting factor which induces the local adaptation of neighboring microvilli. This is indicated by the observation that the maximum response amplitude recorded after light adaptation was approximately equal to that measured in the dark-adapted state when the same number of microvilli was photoactivated (Fig. 12). In other words, in both cases a microvillus excites the receptor to the same degree, in the dark-adapted state by absorption of a single quantum and in the light-adapted state by ~1,000 quantum absorptions (compare Fig. 2). This equality could only be manifest if in the light-adapted state the factors leading to receptor excitation compensate those that desensitize the receptor.

In summary, the results presented in this article support the hypothesis that the microvilli are the elementary functional units in insect photoreceptors. This hypothesis has been proposed originally for receptor excitation (Hamdorf, 1979), and recently gained support by a quantitative analysis of photoreceptor signal/noise ratio in *Lucilia cuprina* (Howard et al., 1987). The results presented here extend the hypothesis, in that the microvilli not only represent the elementary units of receptor excitation but also of receptor adaptation.

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