Measurement of Cytosolic Ca\(^{2+}\)
Concentration in *Limulus* Ventral
Photoreceptors using Fluorescent Dyes

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ABSTRACT Several Ca-sensitive fluorescent dyes (fura-2, mag-fura-2 and Calcium Green-5N) were used to measure intracellular calcium ion concentration, \(C_{ai}\), accompanying light-induced excitation of *Limulus* ventral nerve photoreceptors. A ratiometric procedure was developed for quantification of Calcium Green-5N fluorescence. A mixture of Calcium Green-5N and a Ca-insensitive dye, ANTS, was injected in the cell and the fluorescence intensities of both dyes were used to calculate the spatial average of \(C_{ai}\) within the light-sensitive R lobe of the photoreceptor. In dark-adapted photoreceptors, the initial \(C_{ai}\) was 0.40 ± 0.22 μM (SD, \(n = 7\)) as measured with fura-2. \(C_{ai}\) peaked in the light-sensitive R lobe at 700–900 ms after the onset of an intense measuring light step, when the spatial average of \(C_{ai}\) within the R lobe reached 68 ± 14 and 62 ± 37 μM (SD, \(n = 5\)) as measured with mag-fura-2 and Calcium Green-5N, respectively. The rate of \(C_{ai}\) rise was calculated to be \(\approx 350 \mu M/s\) under the measuring conditions. The resting level of Mg\(^{2+}\) was estimated to be 1.9 ± 0.9 mM, calculated from mag-fura-2 measurements. To investigate the effect of adapting light on the initial \(C_{ai}\) level in the R lobe, a 1-min step of 420 nm background light was applied before each measurement. The first significant (\(P < 0.05\)) change in the initial level of \(C_{ai}\) occurred even at the lowest adapting light intensity, which delivered \(\approx 3 \times 10^5\) effective photons/s. The relative sensitivity of the light-adapted photoreceptors was linearly related to the relative \(C_{ai}\) on a double log plot with slope between −4.3 and −5.3. We were unable to detect a \(C_{ai}\) rise preceding the light-activated receptor potential. The \(C_{ai}\) rise, measured with Calcium Green-5N, lagged 14 ± 5 ms (SD, \(n = 32\)) behind the onset of the receptor potential at room temperature in normal ASW. In the absence of extracellular Ca\(^{2+}\) and at 10°C, this lag increased to 44 ± 12 ms (SD, \(n = 17\)).

INTRODUCTION

A rise in intracellular calcium, \(C_{ai}\), accompanies depolarization of *Limulus* ventral photoreceptors during transduction (Brown and Blinks, 1974). Elevation of \(C_{ai}\) both excites and adapts ventral photoreceptors (Lisman and Brown, 1972; Payne, Corson, and Fein, 1986). In *Limulus* ventral photoreceptors, the luminescent photoprotein,
aequorin (Brown and Blinks, 1974), the absorbance Ca-indicator dye arsenazo III (Brown, Brown, and Pinto, 1977) and calcium-sensitive microelectrodes (Levy and Fein, 1985) have all been used to estimate Cai. These studies indicate that after a bright flash, Cai reaches extraordinarily high levels, in the range 30-100 μM. However, there are discrepancies between the estimates of Cai given by the various methods. For instance, the value of Cai in darkness estimated using the photoprotein aequorin, 0.66 ± 0.09 μM (O'Day and Gray-Keller, 1989), is quite different from the estimate of 3.5 ± 2.5 μM obtained using calcium-sensitive electrodes.

Several Ca-sensitive fluorescent dyes are now available which might be used in combination to measure Cai over a wide range in Limulus photoreceptors (Gryniewicz, Poenie, and Tsien, 1985; Raju, Murphy, Levy, Hall, and London, 1989; Haugland, 1992). The advantage of using these dyes is the rapidity and simple stoichiometry of their reaction with calcium ions. The main limitation is that ventral photoreceptors respond to the wavelengths of light used to stimulate the dyes. Cai can, therefore, only be estimated during the response of the photoreceptor to the illumination used to excite the dyes. In the present paper we address the following questions. First, what is the peak value of Cai during a bright flash and which of the dyes most accurately estimates the spatial average of Cai within the photoreceptor's light-sensitive R lobe? Second, what is the value of initial Cai at the onset of measuring light both in dark-adapted cells and in the presence of an adapting background light? Third, does the use of fluorescent indicators confirm the conclusion of aequorin and arsenazo III studies that the electrical response precedes any detectable rise in Cai (Payne and Flores, 1992; Stieve and Benner, 1992).

MATERIALS AND METHODS

Apparatus

Photoreceptors situated on the side of the ventral optic nerve were viewed through a Nikon Diaphot inverted microscope and impaled with a conventional micropipette. The temperature of the chamber containing the ventral nerve was maintained by passing the superfusing artificial sea water (ASW) through a Peltier device (Interconnection Products Inc., Pompano Beach, FL) at a rate of ~1 ml/min. A miniature copper-constantan thermocouple placed in a glass micropipette was inserted in the bath as close to the nerve as possible and connected to digital thermometer (DP30, Omega Inc., Stamford, CT). The electrical response of the cell to light was acquired simultaneously with the optical signal at the rate of 200 Hz using the second channel of the microfluorimeter A/D board so that any synchronization problems were eliminated. We also checked the timing independently by placing a photocell in the specimen plane of the fluorimeter. The photocell signal was connected to the A/D input usually reserved for membrane potential. Light reflected from the photocell was collected by the photomultiplier. When the shutter for the fluorescence excitation light was activated, comparison of the photocell trace with the photomultiplier measurement revealed no difference in rise time, beyond the 5-ms integration time. No timing correction for this integration time was made in displaying the fluorescence traces.

The number of effective photons delivered per receptor per second, by incident light, was estimated by using calibrated neutral density (ND) filters to attenuate the light until individual light-evoked quantal events could be counted (Yeandle and Spiegler, 1973).

A conventional epillumination system for ratio-fluorescence microscopy was used to detect dye fluorescence (Deltascan; PTI Inc., South Brunswick, NJ). The incident light excited both
the photoreceptor and the dye, producing $\sim 4 \times 10^7$ photoisomerisations of rhodopsin/s at 340 nm and $10^9$ photoisomerisations/s at 500 nm. From previous studies (O'Day and Gray Keller, 1989; Levy and Fein, 1985), we expect both of these flashes to saturate the calcium-release mechanism and produce maximal elevations of $Ca_{\text{ii}}$. A shutter (Uniblitz, Vincent Associates, Rochester, NY) with a time to fully open of 6 ms controlled the incident illumination. Chopping between wavelengths for fura-2 and mag-fura-2 measurements occurred at a maximum rate of one 340/380 nm cycle every 5 ms. The same acquisition rate was used for Ca Green-5N. Using a higher acquisition rate increased the measuring error due to photon shot noise. A 20x lens (Nikon Flu o ×20, 0.75 NA) was used to collect fluorescence which was measured with a photon-counting photomultiplier. Photon counts from the photomultiplier were placed in time bins and stored with the electrical recording on a computer. Before being focused onto the photomultiplier, an intermediate image of the cell was created by a lens system (Deltascan D1040). This image could be viewed using an eyepiece, and a mask could be placed over the image plane, restricting the measurement of fluorescence to the light-sensitive rhabdomeral (R)-lobe of the photoreceptor. The positioning of the mask over the R-lobe was verified by placing a red light-emitting diode behind the mask and moving it across the cell until the region with maximal sensitivity was found. In most experiments, the area from which fluorescence was collected did not exceed $20 \times 20 \mu m$.

For experiments in which a background adapting light accompanied the light used to excite the dye and photoreceptor, the light emerging from the excitation monochromators of the fluorimeter was combined by a beam splitter with 420 nm light from a Xenon arc lamp, equipped with an electromechanical shutter and attenuated by neutral density and heat filters.

Pressure injection of fluorescent dyes into the cells was achieved as previously described (Corson and Fein, 1983). Dye was delivered by a series of pressure pulses delivering a total of 10–100 pl of fluid over a period of $\sim 30$ min. The volume of fluid delivered by any one pressure pulse did not exceed 10 pl into a cell having a total volume of $\sim 500$ pl (Corson and Fein, 1983). The physiological performance of the cells was continuously monitored throughout the injection procedure. Only the cells capable of producing single photon events after the injections were considered for further experiments. After injection of 10–100 pl of dye solution, at least 5 min was allowed for equilibration before measurement of fluorescence. The net loss of fluorescence after this time due to leakage of dyes, diffusion from the photoreceptor cell body and bleaching was $\sim 20\%$/h for all of the dyes used.

Chemicals and Solutions

Potassium salts of fura-2 (Grynkiewicz et al., 1985), mag-fura-2 (furaptra; Raju et al., 1989) and Calcium Green-5N (Haugland, 1992), and the sodium salt of ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid) were obtained from Molecular Probes Inc. (Eugene, OR). Di-bromo-BAPTA was obtained from Calbiochem Corp. (La Jolla, CA). Materials for constructing calcium-sensitive macroelectrodes were obtained from Fluka Inc. (Ronkonkoma, NY). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). When necessary, contaminating Ca$^{2+}$ was removed from solutions by passing them through a column of BAPTA polystyrene ("Calcium Sponge S," Molecular Probes Inc.). Calcium-sensitive dyes injected into cells were dissolved in a solution containing 100 mM potassium aspartate, 10 mM Hapes, pH 7.0. ASW contained (in millimolar) 435 NaCl; 10 KCl; 20 MgCl$_2$; 25 MgSO$_4$; 10 CaCl$_2$; 10 mM HEFES, pH 7.0. 0 Ca-ASW contained the above, without CaCl$_2$ and with 1 mM K$_2$EGTA added.

Properties of Ca-sensitive Dyes

The $K_d$ for Ca$^{2+}$ of each dye was first established for solutions containing 400 mM KCl at pH 7.0. This relatively high ionic strength was chosen to approximate the intracellular ionic
environment known to exist in neurons of other marine species (Hodgkin, 1951; O'Day and Phillips, personal communication). For fura-2, a $K_d$ for Ca$^{2+}$ of $0.642 \pm 0.149 \mu M$ (SD; $n = 7$) was obtained by titration (Fig. 1 A), using mixtures of two solutions containing 1 $\mu M$ fura-2, 400 mM KCl, 10 mM K-MOPS, pH 7.0 and either 10 mM EGTA or 10 mM CaEGTA. The [Ca$^{2+}$] in the mixed solutions was calculated using a $K_d$ for EGTA of 0.575 $\mu M$ in 400 mM KCl, pH 7.0 (Harafuji and Ogawa, 1980) and confirmed by using a Ca-sensitive macroelectrode (Amman, Buehrer, Schefer, Mueller, and Simon, 1987).

For mag-fura-2, a $K_d$ for Ca$^{2+}$ of $56 \pm 8 \mu M$ (SD; $n = 5$) was obtained by titration (Fig. 1 A), using mixtures of two solutions containing 1 $\mu M$ mag-fura-2, 400 mM KCl, pH 7.0 with or without 10 mM CaCl$_2$. The nominally Ca-free solution was prepared without addition of EGTA. We also obtained a $K_d$ for Mg$^{2+}$ of $4.7 \pm 0.5$ $\mu M$ (SD; $n = 3$) by titration (Fig. 1 A) using mixtures of a solution containing 1 $\mu M$ mag-fura-2, 400 mM KCl, 10 mM K-MOPS, pH 7.0 and a solution containing 1 $\mu M$ mag-fura-2, 100 mM KCl, 10 mM K-MOPS, 100 mM MgCl$_2$, pH 7.0.

For Calcium Green-5N, a $K_d$ for Ca$^{2+}$ of $67 \pm 8 \mu M$ (SD; $n = 5$) was obtained by titration (Fig. 1 B, closed triangles), using mixtures of two solutions containing 1 $\mu M$ Calcium Green-5N,
400 mM KCl, 10 mM K-MOPS, pH 7.0 with or without 10 mM CaCl₂. As in case of mag-fura-2, Ca-free solution was not buffered with EGTA. At [Ca²⁺] < 10 μM, the Calcium Green-5N fluoresced more than expected from a single site model of Ca binding. We confirmed this deviation by extending the results to calcium concentrations below 3 μM (Fig. 1 B, open triangles) using solutions whose calcium concentration was buffered with 10 mM di-bromo BAPTA (K_d = 5 μM in 400 mM KCl). Calcium Green-5N fluorescence was insensitive to Mg²⁺ (Fig. 1 B). The ability of Mg²⁺ to displace Ca²⁺ and reduce Calcium Green-5N fluorescence in the presence of Ca²⁺ was assayed by adding MgCl₂ to a solution containing 50 μM CaCl₂, 1 μM Calcium Green-5N, 400 mM KCl, 10 mM K-MOPS, pH 7.0. Addition of 2 mM MgCl₂, the approximate intracellular [Mg²⁺], had no significant effect (P < 5%) on the fluorescence of this solution. We therefore ignored the influence of Mg²⁺ in our calibrations of Ca⁻. Addition of > 10 mM MgCl₂ progressively reduced the fluorescence. In two such experiments, K_d's for Mg²⁺ of 46 and 61 mM respectively were calculated by fitting data to a model in which Ca²⁺ and Mg²⁺ compete for a single binding site.

Calibration of Fura-2 and Mag-Fura-2 Signals

Calibration of fluorescence was achieved by reference to external standard solutions. At the end of every experiment, droplets of 1 μM dye, 400 mM KCl, 10 mM K-MOPS pH 7.0, containing either 10 mM EGTA or 10 mM CaCl₂ were placed on a standard microscope cover slip glass. The same type of the cover glass was used throughout the experiments and calibration procedures and therefore its effect on the spectral measurements could be omitted. Fluorescence at 340 and 380 nm was obtained for this solution and for a solution containing no dye using the same microscope lens and viewing aperture as for the cell. The resulting maximum and minimum ratios of 340/380 nm fluorescence (R_min and R_max) and the K_d's described above, were used to calculate the calcium concentrations from the 340/380-nm ratios obtained from cellular measurements according to the method described by Grynkiewicz et al. (1985). The cellular measurements were corrected for background by subtraction of the fluorescence observed through the same aperture from the R lobes of neighboring cells not filled with dye. For some cells, background was also assayed by treating the cells for 30 min. with 50 μM 4-Br-A23187 in 1% DMSO followed by the addition of 1 mM MnCl₂ to quench fura-2. Autofluorescence was then detected exactly in the measuring area. No significant differences were found between these two methods of background correction. The autofluorescence was typically 5-20% of the maximum cellular signal and did not change significantly during the 4.8-s measurement period. After correction, the changes in 340 nm fluorescence compared to the changes in 380 nm fluorescence obtained in vivo were less than those expected from the in vitro calibrations. This difference has been ascribed by others to enhanced 380 nm fluorescence due to the interaction of the dye with high viscosity cellular constituents (Poenie, 1990). Therefore, a so-called “viscosity correction” factor was calculated for each cell and applied to the calibration procedure as described by Poenie (1990). Typical values of the calibration constants for fura-2 (using the nomenclature of Grynkiewicz et al., 1985) were R_max = 0.29; R_min = 0.22; S_x/S_y = 13.1, viscosity correction factor = 0.6. Typical values for mag-fura-2 were R_max = 12.9, S_x/S_y = 28.4. No viscosity correction was needed for mag-fura-2.

Calibration of Calcium Green-5N Signals

To calibrate Calcium Green-5N signals, we added 10 mM of the Ca-insensitive dye ANTS to 250 μM Calcium Green-5N and injected the mixture into cells. ANTS was chosen for its insensitivity to changes of pH and its highly polar nature, which prevents binding to membranes (Ellens, Bentz, and Skoka, 1985). ANTS was excited at 360 nm and emission peaked at 514 nm. Therefore, a standard fura-2 filter cube (Omega Optical Inc., Brattleboro,
VT) having 510-nm bandpass filter could be used for measuring ANTS fluorescence. To measure light-induced changes in Calcium Green-5N fluorescence, a 4.6-s, 500-nm exciting light step was delivered through a standard fluo-3 filter cube set (Omega Optical Inc.). An ANTS fluorescence excitation spectrum was determined immediately after each measurement of Calcium Green-5N fluorescence. The ratio, \( R \), of 500 nm fluorescence from Calcium Green-5N to 360 nm fluorescence from ANTS was determined after subtraction of the appropriate backgrounds. Calibration was achieved by comparing this ratio to that of the minimum and maximum ratios determined in droplets containing 1 \( \mu \)M Calcium Green-5N, 40 \( \mu \)M ANTS, 400 mM KCl, 10 mM K-MOPS pH 7.0 and either 10 mM EGTA or 10 mM CaCl\(_2\), placed on a microscope cover slip glass. The titration data of Fig. 1B were then used to construct a curve that related the quantity \( \frac{(R-R_{\text{min}})}{R_{\text{max}}} \) to calcium concentration. Fig. 2A (solid lines) illustrates ANTS and Calcium Green-5N spectra recorded in droplets containing EGTA and CaCl\(_2\). An ANTS spectrum recorded inside a cell (Fig. 2A, dashed line) and the corresponding change in Calcium Green-5N fluorescence from inside the cell upon illumina-

![Figure 2. Use of ANTS to calibrate Calcium Green-5N signals. (A) Excitation spectra (solid lines) of a solution containing 40 \( \mu \)M ANTS, 1 \( \mu \)M Calcium Green-5N and either 10 mM CaCl\(_2\) or 1 mM EGTA in 400 mM KCl, 10 mM MOPS, pH 7.0. The spectra were obtained from droplets placed on the glass cover slips using either the fluo-3 dichroic cube set (500 nm excitation, 505 nm emission: Calcium Green-5N) or the fura-2 dichroic cube set (360 nm excitation, 510 nm emission: ANTS). The ANTS spectra with and without Ca\(^{2+}\) coincide. Also plotted is the excitation spectrum (dots) of ANTS recorded from the R-lobe of a Limulus photoreceptor injected with a solution containing 10 mM ANTS and 250 \( \mu \)M Calcium Green-5N. (B) Time course of fluorescence changes due to Calcium Green-5N, observed after intense excitation of the same photoreceptor as used in A by a 4.6 s flash of 500 nm light, beginning at the start of the trace.

Underestimation of Transient Changes in Ca\(_i\) Due to Saturation of Fura-2

Calcium release in Limulus ventral photoreceptors is initiated by events at the microvillar plasmalemma. Electron micrographs reveal a calcium-storing endoplasmic reticulum within a micron of the microvilli (Calman and Chamberlain, 1982; Payne et al., 1988).

Given that our dye measurements are made from a substantial volume of R lobe, we cannot estimate Ca\(_i\) within this subplasmalemmal region, only the spatial average of Ca\(_i\) within the R
lobe. Our estimates of the spatial average of \( \text{Ca}_i \), however, are subject to a systematic error if saturation of dye occurs in the subplasmalemmal region. To investigate the possible magnitude of this error, we constructed a simple model in which release of \( \text{Ca}^{2+} \) occurs only within the outermost 1 \( \mu \)m shell of a 60-\( \mu \)m diam sphere representing the R lobe (Fig. 3A). Each shell initially contained 1 mM of a slow Ca-buffer, B, and 100 \( \mu \)M fura-2 or mag-fura-2. In each shell, the following interactions between dye, buffer and \( \text{Ca}^{2+} \) occurred.

\[
\begin{align*}
& k_1 & [\text{Ca}^{2+}] + [B] & \rightleftharpoons [\text{Ca}.B] \\
& k_2 & \\
& k_3 & [\text{Ca}.dye] + \text{Ca}^{2+} & \rightleftharpoons [\text{Ca}.dye] \\
& k_4 & \\
& K_v & 400 \text{ nM} \\
& K_r & 400 \text{ nM} \\
& D_{\text{Ca}} & 10^{-7} \text{ cm}^2 \text{s}^{-1} \\
& D_{\text{dye}} & 5 \times 10^{-7} \text{ cm}^2 \text{s}^{-1} \\
& D_{\text{Ca}.dye} & 5 \times 10^{-7} \text{ cm}^2 \text{s}^{-1} \\
\end{align*}
\]

The values of \( k_1, k_2, k_3, k_4 \) and the diffusion constants for movement of \( \text{Ca}^{2+}, \text{dye} \) and \( \text{Ca}.\text{dye} \) between shells as well as the initial concentrations of the various reactants are given in Fig. 3. Values for the \( K_v \)s of the dyes are those found experimentally.

The rate constants, \( k_3 \) and \( k_4 \) for binding and release of \( \text{Ca}^{2+} \) by the dyes were both reduced by a factor of 10 from published values (Baylor and Hollingworth, 1988) to avoid requiring an
excessively short time step for the integration and so to keep the computing time within reasonable bounds. Diffusion constants for the dyes are consistent with published estimates for fura-2 (Timmerman and Ashley, 1986). The characteristics of the slow buffer, B, were chosen to simulate the uptake of Ca$^{2+}$ into a uniformly distributed network of endoplasmic reticulum with an apparent affinity for Ca$^{2+}$ of 400 nM (Walz, 1982) and to provide an elevation of Ca$^{2+}$ of approximately the time course and magnitude reported by mag-fura-2 and Calcium Green-5N. Given the chosen values of $k_l$ and the concentration of B, the rate of binding of Ca$^{2+}$ by B at a [Ca$^{2+}$] of 1 μM would be 0.005 μmol l$^{-1}$ ms$^{-1}$, about six times slower than that reported for uptake into endoplasmic reticulum of nerve terminals at 30°C (Rasgado-Flores and Blaustein, 1987).

Rates of change of Ca$_i$, B, Ca.B, dye and Ca.dye were calculated within each shell using a set of differential equations which described Eqs. 1 and 2 as well as the diffusional fluxes of Ca$^{2+}$, dye and Ca.dye between neighboring shells. The differential equations describing the fluxes of the reactants within each shell were integrated using the Euler method with a time step of 0.1 ms. Initially, the Ca$_i$ was set at 250 nM, distributed uniformly through the sphere, with concentrations of the other reactants calculated so as to be in equilibrium with the Ca$_i$. After 50 ms of integration, 4.6 × 10$^{10}$ Ca$^{2+}$ ions/s were added to the outermost 1 μm shell for 350 ms in order to simulate a localized, transient release of Ca$^{2+}$ ions. We also performed simulations in which the sphere was divided into 0.1-μm shells and the time steps were reduced to 0.01 ms. For these simulations, the same addition of Ca$^{2+}$ ions was made to the outermost 0.1-μm shell. As might be expected, the predicted dye signals did not substantially differ from that predicted by the first model for times greater than 10 ms.

The estimate of the spatial average of Ca$_i$ within the R lobe calculated from the fura-2 signal (Fig. 3 B) greatly overestimates the true spatial average within the model R lobe at early times and shows a much delayed time to peak. The estimate of Ca$_i$ calculated from the mag-fura-2 signal more closely follows the true spatial average within the R lobe (Fig. 3 C), while still somewhat underestimating the peak. The model illustrates how a transient, localized release of calcium might account for the differences in the rate of rise and time to peak of Ca$_i$ as estimated by fura-2 and mag-fura-2, with much less difference between their estimates of Ca$_i$ at later times. We conclude that although fura-2 undergoes the larger absolute change in fluorescent intensity, it cannot be used to quantify Ca$_i$ during the response to a bright flash.

RESULTS

Fura-2 Provides an Estimate of Ca$_i$ in the Dark-adapted Photoreceptors

Photoreceptors were injected with 10–100 pl of a solution containing 500 μM fura-2. The concentration of fura-2 that was injected was chosen based on previous work in which injections of the solutions containing 500 μM of the calcium chelator EGTA (Fein, Payne, Corson, Berridge, and Irvine, 1984) or 1 mM BAPTA (Levy and Payne, 1993) did not appear to affect the ability of the calcium-releasing agent inositol 1,4,5 trisphosphate to excite or adapt the photoreceptor and are therefore unlikely to interfere with the effect of released calcium on phototransduction. Cells were then dark adapted for up to 20 min, until single photon events of amplitude 1–5 mV were observed. A 4.6-s step of rapidly alternating 340/380 nm light, producing $\sim 4 \times 10^7$ photoisomerisations/s, was then delivered to the cell. After the onset of this illumination, fluorescence to 380 nm light dropped and fluorescence to 340 nm light increased as the electrical response of the cell to the flash proceeded (Fig. 4). The increased noisiness of the Ca signal at the peak of the response is a result of dye saturation. Our model calculations (see Methods) lead us to believe that local
saturation of fura-2 prevents meaningful calibration of the calcium signal once calcium release begins. We therefore present the signal as the ratio of fluorescence at 340 nm and 380 nm and confine quantification of \( \text{Ca}_i \) to the period before significant calcium release was observed.

During the latent period of the electrical response to light, between 10 and 50 ms after the shutter opened, the 340/380 nm ratio remained unchanged at 0.44 ± 0.04 \( \mu \text{M} \), corresponding to a \( \text{Ca}_i \) of 0.35 ± 0.03 \( \mu \text{M} \) \( \text{Ca}_i \) (mean ± SD) (Fig. 8 C). We assume that this value also represents the resting level of \( \text{Ca}_i \) in this cell. Measurements in seven other cells produced a \( \text{Ca}_i \) of 0.40 ± 0.22 \( \mu \text{M} \). In four other cells, treatment with 0\( \text{Ca} \)-ASW for more than 10 min resulted in a reversible decrease in \( \text{Ca}_i \) from 0.46 ± 0.22 \( \mu \text{M} \) to 0.2 ± 0.12 \( \mu \text{M} \) within 10 min.

\( \text{Ca}_i \) during Steady Background Illumination

The 340/380 nm illumination used to measure \( \text{Ca}_i \) was superimposed upon an adapting background of 420 nm light, which began 1 min before measurement of \( \text{Ca}_i \). The unattenuated 420 nm light source produced \( \sim 3 \times 10^6 \) photoisomerisa-
tions/s. The wavelength of the adapting light was chosen so as to neither excite fura-2 significantly, nor to pass the 510-nm bandpass filter used in the pathway through which fluorescence was detected. We did not find any significant effect of the adapting light on the detection of fura-2 fluorescence.

Fura-2 signals were recorded and averaged during the latent period of the electrical response to the 340/380 nm flash. Calculated estimates of Ca_i during the latent period for different intensities of the adapting light are summarized in Table I. We assume that these values represent the level of initial Ca_i in the presence of the adapting background alone. At all intensities, the indicated levels of Ca_i were well below those which might saturate the fura-2 and we therefore expect the calculated values of Ca_i to reflect the spatial average value within the R lobe. In three of four cells exposed to the entire range of adapting light intensities, the first significant (P < 0.05) change in Ca_i occurs at the lowest adapting light intensity step. We estimate that this light intensity produces ~ 3 × 10^3 photoisomerisations/s.

| Cell number | Sensitivity | Intensity of the adapting light, logI | Ca_i, μM ± SD |
|-------------|-------------|--------------------------------------|---------------|
|             | -logI       | -3        | -2  | -1  | 0       |
| 1           | 5.8         | .128 ± .025 | .357 ± .074 | .390 ± .062 | .766 ± .189 | 1.138 ± .369 |
| 2           | 5.5         | .553 ± .191 | .750 ± .197 | .740 ± .209 | 1.033 ± .410 | 2.202 ± .512 |
| 3           | 5.0         | .409 ± .166 | .884 ± .304 | .862 ± .258 | 1.619 ± .777 | 2.811 ± 1.904 |
| 4           | 5.8         | .210 ± .079 | .157 ± .074 | .260 ± .074 | .447 ± .180 | .736 ± .387 |
| Average     | .325 ± .192 | .532 ± .341 | .563 ± .284 | .966 ± .496 | 1.721 ± .915 |

**Relationship between Ca_i and Sensitivity to Light**

The sensitivity of the photoreceptor was assayed in the presence of the adapting background light using brief flashes from a third light beam. We used white light from a 100-W halogen lamp combined with the 420-nm adapting light by a beam splitter. The sensitivity of the photoreceptor was expressed as the negative log of light intensity that produced a criterion light response of 10 mV. For a given cell, sensitivity fell as background light intensity and Ca_i increased (Fig. 5). When plotted using double logarithmic axes, the relationship between sensitivity relative to the dark-adapted state and Ca_i could be fitted by a linear regression with slope of -4.33 (Fig. 5 B). Three other cells showed very similar relationships of the sensitivity versus Ca_i (mean slope = -5.12, mean r = 0.91). We note, however, that although Ca_i and sensitivity relative to the dark-adapted value were closely correlated in a given cell, the considerable scatter of our estimates of Ca_i in darkness between different cells was not closely correlated with the absolute sensitivity of each dark-adapted cell to light (Table I).
Mag-Fura-2 Provides an Estimate of Resting Mg, and the Elevation of Ca, during Illumination

Photoreceptors were injected with 10–100 pl of a solution containing 500 μM mag-fura-2. Cells were then dark adapted for up to 20 min, until 1–5 mV responses to single photons were observed. A 4.6-s flash of rapidly alternating 340/380 nm light, producing ~4 × 10^7 photoisomerisations/s was then delivered to the cell. Unlike the response of fura-2, the response of mag-fura-2 shows an initial transient component during which 380-nm fluorescence drops to 47% of the peak and indicating no saturation of the total dye (Fig. 6). For the cell of Fig. 6, if the effect of Mg^{2+} on dye fluorescence is ignored, an estimate of resting Ca, of 18 μM is obtained.
To reconcile this estimate with the value of 0.4 μM estimated using fura-2, a value for resting Mg₂⁺ of 1.5 mM must be assumed. For five other cells the estimate of resting Mg₂⁺ was 1.9 ± 0.9 mM assuming resting Caᵢ to be 0.4 μM.

The estimate of Caᵢ shown in Fig. 6 assumes that Mg₂⁺ does not change significantly during the response to light. The estimate of Caᵢ peaks at ~80 μM, 450–600 ms after light onset. By the end of the illumination period, Caᵢ in this cell dropped to ~13 μM (Fig. 6). The initial rate of rise of the elevation of the spatial average of Caᵢ is ~400 μM/s, corresponding to 2.4 × 10¹⁰ Ca²⁺/s within the ~100 pl vol of the R lobe, or ~600 Ca²⁺ per effectively absorbed photon. This value is likely to be an underestimate of the number of Ca²⁺ ions released per photon, due to absorption of Ca²⁺ ions by cellular buffering mechanisms. Several features of the light-induced elevation of Caᵢ in five cells were quantified and are tabulated (Table II) for comparison with the data obtained using Calcium Green-5N.

**Calcium Green-5N Provides a Similar Estimate of the Elevation of Caᵢ during Illumination to that Provided by Mag-Fura-2**

We wished to measure Caᵢ using another indicator with low affinity for Ca²⁺ but which was insensitive to changes in Mg²⁺. Calcium Green-5N possesses the required
sensitivity Ca\(^{2+}\) and insensitivity to Mg\(^{2+}\) (Fig. 1 B) but does not change its fluorescence spectrum upon binding calcium and hence cannot be used for ratio measurements of Ca\(_i\). However, we identified a water soluble, calcium-insensitive dye, ANTS, which absorbs in the UV far from the excitation spectrum for Calcium Green-5N and hence can be used as a measure of the amount of coinjected Ca indicator. The ratio of ANTS to Calcium Green-5N fluorescence was therefore used to estimate Ca\(_i\) (see Methods). Photoreceptors were injected with 10–100 pl of a solution containing 250 \(\mu\)M Calcium Green-5N and 10 mM ANTS. Cells were then dark adapted for up to 20 min, sufficient to produce detectable responses to single photons. A 4.6-s step of 500 nm light, producing \(\sim 10^9\) photoisomerisations/s was then delivered to the cell. An estimate of Ca\(_i\) is illustrated in Fig. 7. Estimated Ca\(_i\) peaks at \(\sim 75\ \mu\)M, 450 ms to 1.5 s after light onset. By the end of the illumination period, Ca\(_i\) in this cell dropped to \(\sim 20\ \mu\)M. Note that estimates of Ca\(_i\) calculated from Calcium Green-5N measurements are not significantly different from the estimates based on mag-fura-2 measurements (Table II).

**TABLE II**

| Dye         | Peak Ca\(_i\) | Final Ca\(_i\) | Initial dCa\(_i\)/dt | Time to peak Ca\(_i\) |
|-------------|---------------|----------------|-----------------------|-----------------------|
| Mag-fura-2  | 68 \(\pm\) 14 | 14 \(\pm\) 3   | 337 \(\pm\) 117       | 0.7 \(\pm\) 0.07      |
| Calcium Green-5N | 62 \(\pm\) 37 | 19 \(\pm\) 9   | 336 \(\pm\) 287       | 0.9 \(\pm\) 0.7      |

Note: all data are quoted as mean \(\pm\) SD (n = 5).

**FIGURE 7.** Use of Calcium Green-5N to estimate Ca\(_i\) during the response of a dark-adapted ventral photoreceptor to intense light. *(Top trace)* The spatial average of Ca\(_i\) within the R lobe, estimated from Calcium Green-5N and ANTS fluorescence as described in the text. The shutter controlling the exciting light began to open at 0.2 s, 10 ms before the start of the traces. *(Bottom trace)* Electrical response of the cell to the flash.
The Electrical Response to Light Precedes the Detection of a Significant Elevation of $\text{Ca}_i$

For all of the dyes, the detection of a significant elevation of $\text{Ca}_i$ lagged 10–20 ms behind the onset of the light-induced depolarization at room temperature (Fig. 8). In investigating this lag further we chose to use Calcium Green-5N because of its small kinetic delay in equilibrating with a change in $\text{Ca}_i$ (∼30 µs in vitro at 17°C and in 100 mM KCl; Vergara and Escobar, 1993) and its insensitivity to Mg$^{2+}$. We injected the photoreceptors with 500 µM Calcium Green-5N alone, without adding ANTS. We define the term “calcium signal” as meaning any rise in Calcium Green-5N fluorescence above its initial background level. To determine the latency of this calcium signal and of the electrical response, we calculated the mean and SD of the calcium and the electrical signals during the first 20 ms after the full opening of the shutter. The first subsequent samples exceeding the mean value by twice the SD determined the latencies of each signal (dashed lines, Figs. 9 and 10). The resolution of our determinations of latency was restricted by the sampling rate of 5 ms (see Methods). To facilitate dark adaptation after each measuring flash, we used measuring flashes of duration 0.5 s, followed by at least 10 min in the dark.

**Figure 8.** Initial timing of electrical responses and elevations of $\text{Ca}_i$. Initial portions of data from Figs. 4, 6, and 7 (dots), showing estimates of $\text{Ca}_i$ (dots) obtained using mag-fura-2 (A), Calcium Green-5N (B) and the 340/380 nm ratio using fura-2 (C) and electrical responses (lines) during intense illumination. The shutter controlling the exciting light began to open at 0.2 s (black box), 10 ms before the start of the traces.
We addressed two questions. First, could we assess quantitatively the timing of the calcium signal and, second, in what manner would temperature and extracellular calcium affect the latent periods of the electrical and calcium signals. At 18°C and in normal ASW, the latency of the calcium signal lagged 14 ± 5 ms (mean ± SD) after the beginning of depolarization (Fig. 9 A). Reducing the temperature from 18 to 6°C increased the latency of the electrical signal by 3.8-fold and that of the calcium signal 3.4-fold (Table III). Lowering temperature also reduced the rate of rise of both signals (Fig. 9 C). As a consequence of these changes, the delay between the electrical and calcium signals increased to 32 ± 2 ms.

Figure 9. The calcium signal (dots) lags the electrical response (lines) after intense 500 nm illumination of ventral photoreceptors filled with Calcium Green-5N. The shutter controlling the exciting light began to open at 0.2 s (black box), 10 ms before the start of the traces. (A) Control response of the cell bathed in ASW at 18°C (18°C). (B–D) Show responses obtained when the cells were bathed in 0Ca-ASW at 18°C (18°C, no added Ca²⁺); ASW at 10°C (10°C, no added Ca²⁺); and 0Ca-ASW at 10°C (10°C, no added Ca²⁺), respectively. Each trace of fluorescence and electrical response is the mean of several measurements made under each condition. Fluorescence intensity is calculated as ΔF/ΔF_{0} after correction for background, where F_{0} is the lowest value of the fluorescence at the beginning of the trace. Dotted lines on each graph represent the threshold to determine the latency of calcium signal (see text).

To further increase the delay between the electrical response and the calcium signal, we investigated the effect of bathing the cells in 0Ca-ASW. This treatment has been previously shown to increase the latency of the electrical signal and of the increase in Ca²⁺, measured using aequorin (Payne and Flores, 1992). The decline of resting Ca²⁺ after removal of extracellular calcium has been shown previously to be complete within 10 min (Bolsover and Brown, 1985; Levy and Fein, 1985). We therefore waited at least 10 min before the first measurement in 0Ca-ASW. In some cells, the latencies of the light response and of the rise in Ca²⁺ increased with each successive flash delivered in 0Ca-ASW. To reduce scattering of the latencies in Table III by this effect, we used records acquired only during the first three light flashes after removal of extracellular calcium. Under these conditions, we found that, at 18°C, 0Ca-ASW did not significantly change the delay between the electrical response.
and the detection of an elevation of Ca$_2^+$ (Table III, Fig. 9 B). Neither was the initial rate of calcium release affected (Fig. 9 B). At 10°C, however, the delay between the electrical signal and the detection of a calcium signals increased from 27 ± 8 ms to 44 ± 12 ms (Fig. 9 D).

**TABLE III**

Latencies of the Light Response ($t_{LR}^{LR}$) and Ca$^{2+}$ Release ($t_{Ca}^{Ca}$) at Different Temperatures in Normal and 0Ca-ASW

| Experiment   | Number of cells | $t_{LR}^{LR}$ | $t_{Ca}^{Ca}$ | Delay |
|--------------|-----------------|---------------|---------------|-------|
| Ca, 18°C     | $n = 32$        | 25 ± 3        | 38 ± 4        | 14 ± 5|
| Ca, 15°C     | $n = 8$         | 29 ± 3        | 44 ± 4        | 15 ± 5|
| Ca, 10°C     | $n = 10$        | 51 ± 11       | 77 ± 6        | 27 ± 8|
| Ca, 6°C      | $n = 3$         | 96 ± 10       | 128 ± 8       | 32 ± 2|
| 0-Ca, 18°C   | $n = 8$         | 31 ± 6        | 46 ± 5        | 15 ± 8|
| 0-Ca, 10°C   | $n = 17$        | 49 ± 10       | 92 ± 17       | 44 ± 12|
The Calcium Signal Measured Using Fura-2 also Lags the Electrical Signal

The sensitivity to Ca$_i$ of Calcium Green-5N is a critical issue in interpreting the effects of 0Ca-ASW on the latency of the calcium signal as measured above. Because Calcium Green-5N is relatively insensitive to submicromolar Ca$_i$, we also investigated the latency of the calcium-signal using fura-2, which has a K$_d$ suitable for measuring small deviations of Ca$_i$ from the resting level in both ASW and 0Ca-ASW. Even though local saturation of fura-2 during calcium release prevents quantitation of Ca$_i$, fura-2 should undergo the largest absolute changes in fluorescent intensity upon calcium release of the dyes we chose to use. For these experiments, we chose to use the ratio of 340/380 nm fluorescence as the calcium signal and used the same definition of latency as for Calcium Green-5N. At 18°C and in 0Ca-ASW the detection of a calcium signal lagged that of the electrical response by 28 ± 9 ms (mean ± SD; n = 4 cells; Fig. 10A). At 11°C and in 0Ca-ASW, the lag of the dye signal latency increased to 43 ± 18 ms (mean ± SD; n = 4 cells; Fig. 10 B). We were therefore unable to demonstrate a shorter lag of the calcium signal in 0Ca-ASW using fura-2, compared to that observed using Calcium Green-5N.

DISCUSSION

Calibration of Fluorescent Dyes

We chose to calibrate our measurements by reference to standard solutions in vitro, rather than attempting to "clamp" Ca$_i$ at different concentrations using ionophores and buffered external calcium concentrations. This choice was prompted by the large size of the ventral photoreceptor and its extensive calcium stores, which would, we felt, make it difficult to adequately control intracellular calcium. Our use of fluorescent dyes to estimate Ca$_i$ is therefore subject to the general uncertainties of the chosen calibration method (Kurebayashi, Harkins, and Baylor, 1993). The choice of ionic strength of our standard calibration solution is critical in determining the K$_d$ of the dyes. For fura-2, estimates of K$_d$ vary from 150 nM (Neher and Augustine, 1992) up to 745 nM (Uto, Arai, and Ogawa, 1991), depending on ionic strength and temperature. The value that we measured and used, 642 ± 149 nM, is similar to values of 745 nM extrapolated from the data of Uto et al. (1991) and 774 or 760 nM used in previous studies of cells from marine invertebrates (Poenie, Alderton, Tsien, and Steinhardt, 1985; Fink, Connor, and Kaczmarek, 1988). A further complication in calibrating the fura-2 signals is the use of a viscosity correction factor. The ratio of the fura-2 fluorescence observed in vivo was always less than expected from the in vitro calibrations. To compensate for this deviation, we followed the method of Poenie (1990) and included a "viscosity correction" factor (Poenie, 1990) in our calibration of fura-2 fluorescence, which reduces the values of the constants $R_{min}$ and $R_{max}$ by a fixed factor and so increases the estimate of Ca$_i$. This correction is purely empirical in our case and has no basis in actual measurements of cytoplasmic viscosity in ventral photoreceptors.

Our calibrations of mag-fura-2 and Calcium Green-5N signals are subject to several uncertainties. For mag-fura-2, the principal assumption is that Mg$_i$ does not change by several millimolar during the light response. Mg$_i$ does not change upon illumina-
tion of barnacle photoreceptors (Rydquist and Brown, 1986). For Calcium Green-5N, the principal assumption is that the coinjected dye, ANTS, distributes within the cytoplasm in a similar manner to Calcium Green-5N and that the fluorescence of both dyes is either unaffected by the cytoplasmic contents or modified to the same degree.

Despite the uncertainties of calibration, the dye measurements present an internally consistent view of $[Ca_{\text{t}}]$. Both mag-fura-2 and Calcium Green-5N indicate that during bright illumination, mean $[Ca_{\text{t}}]$ within the R lobe reaches a peak of 50–100 μM, to fall within 5 s to 10–20 μM.

**Differences between Dye Signals. Effects of Spatial Averaging and Saturation of Fura-2**

It is important to recognize that our estimates of $[Ca_{\text{t}}]$ reflect the spatial average of $[Ca_{\text{t}}]$ within the R lobe or a substantial fraction of the R lobe, a structure ~40–60 μm diam (Calmain and Chamberlain, 1982). It is highly probable that light-induced calcium-release is initiated immediately adjacent to the plasma-membrane, either by virtue of limited diffusion of messengers released by light, such as inositol 1,4,5 trisphosphate, or due to subcellular localization of calcium stores. It is probable that gradients of calcium ions, bound and unbound dye will exist during the first second or so of the response to bright light, so that our estimated spatial average will underestimate the value of $[Ca_{\text{t}}]$ at the site of release. Without performing confocal measurements, we cannot avoid this underestimation. However, there is an additional, avoidable, systematic error in estimating the spatial average of $[Ca_{\text{t}}]$ if the fluorescent dye becomes saturated with calcium at any point within the cell. Our model of calcium release (see Methods) indicates that if calcium release is localized to within 1 μm of the plasma membrane, saturation of fura-2 will greatly reduce the apparent rate of rise of the estimate of $[Ca_{\text{t}}]$ and will retard the time to peak. The calibrated dye signal will only begin to measure the true spatial average of $[Ca_{\text{t}}]$ only when $[Ca_{\text{t}}]$ at the site of calcium release falls below the level which saturates the dye (Tillotson and Nasi, 1988). We believe this to be the cause of the differences between the fura-2 and mag-fura-2 or Calcium Green-5N signals during the first few seconds of the response. We do not, therefore, use fura-2 to estimate levels of $[Ca_{\text{t}}]$ during the peak response to a bright flash, when saturation of the dye occurs. However the resting levels of $[Ca_{\text{t}}]$ and those seen during adapting illumination do not come close to saturation of the dye and therefore are expected to reflect the spatial average of $[Ca_{\text{t}}]$ within the R lobe.

**Comparison with Previous Estimates of $[Ca_{\text{t}}]$ and $[Mg_{\text{t}}]$**

Our estimates indicate that the spatial average of $[Ca_{\text{t}}]$ can increase by more than 100-fold at the peak of the response to bright illumination. Our estimate of $[Ca_{\text{t}}]$ in darkness and in ASW, 0.40 ± 0.22 μM, is somewhat less than the value of 0.66 ± 0.09 μM reported using aequorin (O’Day and Gray-Keller, 1989). However, our estimate differs ~10-fold from the 3.5 ± 2.5 μM obtained using ion-selective electrodes. Our estimate of the peak, light-induced elevation of $[Ca_{\text{t}}]$, 60–70 μM, falls in the range of concentrations reported using other methods, 30 μM (aequorin: O’Day and Gray-Keller, 1989), 44 μM (calcium-sensitive electrodes: Levy and Fein, 1985) and 100 μM (arsenazo III: Brown et al., 1977). Our estimate of resting $[Mg_{\text{t}}]$,
1.9 ± 0.9 mM, is the first in Limulus photoreceptors and it compares to an estimate of 1.7 mM in Balanus photoreceptors, measured with the dye eriochrome blue (Rydqvist and Brown, 1986). We note that the large light-induced elevation of \( Ca_i \) in Limulus photoreceptors precludes the use of mag-fura-2 to monitor any small light-induced changes in Mg.

**Relationship between \( Ca_i \) and Light Adaptation**

Prior illumination by an adapting background increased \( Ca_i \) during the latent period of the response to the flash used to measure fluorescence. We assume that this increase reflects the steady elevation of \( Ca_i \) resulting from the background light alone. As expected from previous studies of Limulus and Balanus photoreceptors (Levy and Fein, 1985; Brown, Rydqvist, and Moser, 1988), the elevation of \( Ca_i \) increased with the intensity of the background light. Even at the highest adapting light intensities, which produced up to \( 10^6 \) photoisomerizations/s, \( Ca_i \) showed no signs of saturation. As noted above, our estimate of resting \( Ca_i \) in darkness is a factor of ~10 less than that reported by Levy and Fein (1985) using calcium-sensitive electrodes. Our estimate of elevations of \( Ca_i \) accompanying prolonged adapting illumination also were less than those of Levy and Fein (1985) by about the same factor of ten. We observed that backgrounds which desensitized the response to light by ~2 log units increased \( Ca_i \) from, for example (Fig. 5) ~0.1 to 0.35 \( \mu \)M. Levy and Fein (1985; Fig. 8) found the same desensitization to be accompanied by an increase from 1 to 3 \( \mu \)M. Therefore, despite the large differences in the absolute values of \( Ca_i \), we agree with Levy and Fein (1985) that large changes in sensitivity accompany relatively small changes in \( Ca_i \) as estimated in any given cell. The log sensitivity of the photoreceptors was linearly related to \( \log[Ca_i] \) with the slopes between -4.3 and -5.3. This relationship is very similar to that observed by Levy and Fein (1985).

Finally, we note that the large scatter of our estimates of \( Ca_i \) in darkness, all from cells producing large quantum bumps and therefore with high sensitivity, contrasts with the close correlation of smaller relative changes of \( Ca_i \) during light adaptation with large reductions of relative sensitivity. Similar variability was noted by Levy and Fein (1985). The significance of this observation for the widely held view that \( Ca_i \) determines sensitivity to light is unclear. Without an independent estimate of the sources of error in determining absolute values of \( Ca_i \), it is impossible to determine whether the scatter in the estimates of absolute values of \( Ca_i \) is an artefact of our measurement technique or whether sensitivity somehow adjusts in any given cell to the absolute level of \( Ca_i \), while retaining a steep dependence on small relative increases in \( Ca_i \).

**Timing of the Electrical and \( Ca_i \) Signals**

Previous studies of the light-induced rise in \( Ca_i \) within Limulus ventral photoreceptors have failed to observe a calcium signal that preceded the electrical response to light (Brown and Blinks, 1974; Brown et al., 1977; Payne and Flores, 1992; Steive and Benner, 1992). The delays of 14–44 ms that we observed between the electrical response and the detection of a \( Ca_i \) signal are not readily explainable by the kinetics of the interaction between \( Ca_i \) and the fluorescent dyes. Mag-fura-2 has been shown in muscle cells to follow elevations of \( Ca_i \) with delays of <1 ms (Konishi, Holling-
worth, Harkins, and Baylor, 1991), Calcium Green-5N responds in vitro with a kinetic delay of < 30 μs (Vergara and Escobar, 1993). Fura-2, as a result of its slower dissociation rate constant, follows calcium transients in muscle with delays of ~ 10 ms at the peak, less at earlier times (Baylor and Hollingworth, 1988). All of these delays are less than those observed.

Large molecules can apparently diffuse, once injected, into the cytoplasmic space beneath the microvilli of ventral photoreceptors (Rubin, Womble, Brown, and Finger, 1989), the space into which Ca²⁺ ions are probably first released. The time course of this diffusion is unknown. To reconcile the observed delays with the proposal that an elevation of Caᵢ is a necessary initiator of the electrical response (Shin et al., 1993), one must propose that the dye in the subrhabdomeral space, even when saturated with Ca, comprises an undetectable fraction of the total fluorescence and that the delay in detection would result from slow diffusion of Ca²⁺ ions out of that space and of dye into it. Certainly, the total microvillar volume (~ 0.8 pl; Fein and Szuts, 1982) is < 1% of the total R lobe volume of ~ 100 pl and dye fluorescence from this area would be indetectable by our fluorimeter. The threshold for detection of a Ca-signal in our experiments using fura-2, for instance, represents a conversion of ~ 7% of the dye to the calcium-bound form. This fraction of the total dye would be contained in the outer 0.72 μm of the R lobe. If calcium release is initiated within 0.1 μm of the plasma membrane, then diffusion delays for the dye and Ca²⁺ to mix in the outermost 0.72 μm layer of the cytoplasm might be expected to be in the range 1 to 20 ms using \( D_{Ca^{2+}} = 2.27 \times 10^{-6} \text{ cm}^2/\text{s} \) for free calcium ions and \( 1.3 \times 10^{-7} \) for calcium ions that interact with binding sites (Albritton, Meyer, and Stryer, 1991). This range should be compared to our experimental values of 14 ± 5 ms using Calcium Green-5N and 29 ± 8 ms using fura-2. Thus, unhindered diffusion of calcium ions is unlikely to account for the delay. However, rapid binding of calcium to a stationary buffer or a diffusion barrier that slows but does not prevent diffusion of dye and Ca²⁺ between the bulk cytoplasm and the submicrovillar space could therefore still account for the observed delays. Further resolution of the delay will require a smaller measurement volume, close to the plasma membrane and the sites of calcium release.

We are greatly indebted to Dr. Peter O'Day and Dr. Enrico Nasi for their valuable comments and helpful criticism.

This work was supported by a grant from National Eye Institute (EY-07743).

Original version received 7 December 1993 and accepted version received 14 September 1994.

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