Journal of Physiology (1991), **433**, *pp.* 467–482
With 7 figures
Printed in Great Britain

ELECTROGENIC Na^+–Ca^{2+} EXCHANGER, THE LINK BETWEEN INTRA- AND EXTRACELLULAR CALCIUM IN THE *LIMULUS* VENTRAL PHOTORECEPTOR

BY ALAN DECKERT AND HENNIG STIEVE

From the Institut für Biologie II, Rheinisch-Westfälische Technische Hochschule Aachen, Kopernikusstrasse 16, 5100 Aachen, Germany

(*Received 14 May 1990*)

SUMMARY

1. *Limulus* ventral photoreceptors were injected with Arsenazo III and the internal change in the calcium concentration, $[Ca^{2+}]_i$, was measured under voltage clamp conditions. It is shown that in response to a light flash the rising phase of the $[Ca^{2+}]_i$ is independent of the clamp voltage, V_m. This observation is contrary to other results reported in the literature. Experiments are reported that resolve this contradiction (see paragraph 4).

2. The relaxation of the $[Ca^{2+}]_i$ after a bright light flash was observed to have a fast and slow phase. A function consisting of the sum of an exponential and a ramp was fitted to the relaxation. The fast phase, characterized by the time constant of the exponential, was observed not to depend on V_m, while the slow phase, characterized by the slope of the ramp, was strongly dependent on V_m. Furthermore the slope of the slow phase is shown to depend on the external Na^+ concentration, but not the time constant of the fast phase.

3. In the dark the $[Ca^{2+}]_i$ was observed to increase when the cell was depolarized and to decrease when the cell was hyperpolarized. This observation was more pronounced when the cell was continuously illuminated.

4. When the cell was clamped to a depolarizing voltage before illumination of the cell, the maximum of the calcium indicator signal was observed to depend on how long the cell had been clamped before applying the light stimulus. This experiment resolves the contradiction mentioned in paragraph 1.

5. The results presented here are consistent with the interpretation that a Na^+–Ca^{2+} exchanger with a stoichiometry greater than 2:1 is the predominant link between intra- and extracellular calcium. Secondly that the light-induced intracellular calcium increase comes from a release by intracellular stores. Finally a measurable uptake of calcium occurs after a light-induced release, possibly by the internal calcium stores. The two-phase recovery of $[Ca^{2+}]_i$ after a light flash is interpreted as being a calcium uptake by the internal stores, the fast phase, and removal by the electrogenic Na^+–Ca^{2+} exchanger, the slow phase.

MS 8493

INTRODUCTION

Calcium has been identified as an important constituent in the phototransduction process of the *Limulus* ventral nerve photoreceptor (see Fein & Charlton, 1977; Fein & Payne, 1989). Several methods have been used to measure the change of internal free calcium ($[Ca^{2+}]_i$) in living cells (see Blinks, Weir, Hess & Prendergast, 1982). These include Arsenazo III (Ar III) which was used in the experiments reported in this paper. The ability of Ar III to measure changes in $[Ca^{2+}]_i$ in *Limulus* ventral photoreceptors was reported in an extensive investigation by Brown, Brown & Pinto, (1977).

If the calcium concentration in the bathing medium, $[Ca^{2+}]_o$, is reduced dramatically and the cell left in the dark, the $[Ca^{2+}]_i$ sinks to below measurable levels (except when using calcium electrodes, see Levy & Fein, 1985), but the cell's calcium response to a single short light stimulus appears nearly normal (Brown & Blinks, 1974; Bolsover & Brown, 1985; Levy & Fein, 1985). In response to a periodic light stimulus programme, the transient increase in $[Ca^{2+}]_i$ gradually vanishes in low $[Ca^{2+}]_o$ (Maaz & Stieve, 1980; Bolsover & Brown, 1985).

Brown & Mote (1974), using voltage clamp, could not show a calcium conductance change in the plasma membrane due to a light stimulus. This and the investigations in low calcium lead to the hypothesis that the light-stimulated calcium increase in the cell originates due to a release from interior sources (Brown & Rubin, 1984; Fein, Payne, Corson, Berridge & Irvine, 1984), although a calcium gradient exists across the plasma membrane favourable to a calcium influx. On the other hand voltage-clamp studies, where the change in $[Ca^{2+}]_i$ and the receptor current were measured simultaneously, indicate that at least part of the light-induced calcium increase is caused by influx of calcium from the extracellular region (Brown & Blinks, 1974; Nagy & Stieve, 1983; Ivens & Stieve, 1984). This discrepancy in the interpretation of the experimental evidence is resolved in this paper.

It has long been suggested that an electrogenic Na^+–Ca^{2+} exchanger exists in the plasma membrane of the *Limulus* ventral photoreceptor (Lisman & Brown, 1972; Ivens & Stieve,1984), similar to that found in other biological preparations (Baker, 1972; Yau & Nakatani, 1984; Hodgkin, McNaughton & Nunn, 1987). O'Day & Keller (1989), who first gave solid evidence for the existence of an electrogenic Na^+–Ca^{2+} exchanger in *Limulus*, noted that the relaxation of the calcium indicator signal occurs in more than one phase. We give a plausible explanation for a two-phase relaxation which we measured using Ar III.

In this paper we show firstly that the rising phase of the light-induced transient increase in $[Ca^{2+}]_i$ is not membrane potential (V_m) dependent. Secondly we demonstrate that at least two processes are responsible for the relaxation of the light-induced $[Ca^{2+}]_i$ increase and finally that the resting $[Ca^{2+}]_i$ level can be influenced by changing V_m. These findings are interpreted in a way that fits the existing data and allows for a better understanding of previous findings.

METHODS

Limulus ventral nerves were dissected under dim white light and after desheathing were fastened to a silicone rubber block (Sylgard 184, Dow-Corning, Midland, MI, USA) and treated with collagenase (2 mg ml^{-1} physiological saline) for 10 min. During the experiments the nerve was

continuously superfused at 15 °C with physiological saline (1–2 ml min^{-1}), pH 7·5, containing (in mM) 485·8 NaCl, 10 KCl, 25 $MgCl_2$, 30 $MgSO_4$, 10 $CaCl_2$ and buffered with 10 mM-HEPES or saline with 50% of the NaCl replaced by LiCl. After switching the saline reservoirs, 90% exchange in the experimental chamber occurred within 2·5–3·5 min, measured photometrically with Fast Green FCF (No. F-7252, Sigma Chemical Co.). The tip of the glass microelectrode was filled with 20 mM-Arsenazo III (lot no. 92f-7386, Sigma Chemical Co.) in 0·5 M-KCl and the rest of the electrode was then back-filled with 0·5 M-KCl solution. A pressure of 1–4 bar was used to inject the Arsenazo III (Ar III) into the cell. The intracellular concentration of Ar III was estimated to be 1 mM within a factor of 2, (see Brown *et al.* 1977; Nagy & Stieve, 1983). The electrodes had resistances of 3–10 MΩ. This procedure is similar to that used by Maaz & Stieve (1980), Nagy & Stieve (1983) and Ivens & Stieve (1984).

Experimental apparatus

The photometric measurements were made by passing white light through a 645 nm interference filter (Schott, 9 nm half-width). The photometric beam was focused on the most active part of the cell, determined by the largest receptor potential, and then into the photomultiplier tube (EMI 9778 B). Another 645 nm interference filter (Schott 8 nm half-width) was placed in front of the photomultiplier tube. When the photometric beam was focused on the cell, the membrane potential increased by 1–8 mV (typically 2–4 mV). The beam spot diameter was approximately 125 μm and had an intensity of 0·01–0·03 mV cm^{-2}. The photometric measurements before injection of Ar III showed no signal. After injection with Ar III an absorption increase was registered in response to a light stimulus given to the photoreceptor. The absorption increase ($\Delta I/I_0$) is attributed to the spectral change of Ar III in the cell as a result of binding Ca^{2+} (Brown *et al.* 1977) which we shall term the Ar III signal. The photomultiplier current was converted to a voltage signal and using a sample-and-hold circuit amplified 100 × over the zero measured at the beginning of the recording period (Nagy & Stieve, 1983). The signals were filtered with a low-pass 100 Hz filter (−3 dB point) (Ortec model 4660 Bandpass Amplifier), to avoid aliasing, before being digitized and stored using a data acquisition system. The data acquisition system consisted of an AT personal computer (EM III/640) with an AD/DA multifunction interface card (PC30, Meilhaus electronic GmbH, Germany). The receptor current or potential was monitored simultaneously with the Ar III signal and filtered (100 Hz) using a single-electrode voltage clamp (SC-100, VF-180 and CA-100 Biologic, France). For an example of the clamp quality, see the first 5 s of the membrane voltage and membrane current in the right-hand side of Fig. 4. The experiments such as that presented in Fig. 3 were also performed using a two-electrode voltage clamp with identical results. All the signals were sampled at 1000 Hz with 12-bit resolution.

For light stimuli, a halogen lamp (Bellaphot, Orsam), controlled by a mechanical shutter or a flash having a 4 ms duration at half-maximum (Metz Mecablitz 60 ct-1) was used. The light stimulus was passed through a wide band filter (Schott BG18, 515 nm maximum) and the intensities regulated by neutral density filters (see figure legends for intensities). The mechanical shutter and the flash trigger were controlled by the data acquisition system.

Data collection and analysis

The following experiments were performed. Receptor currents and Ar III responses, to a bright flash given every minute, were recorded at different V_m's. V_m was held constant during the 10 s recording period and between recording periods the cell was clamped near the resting potential. The pre-stimulus clamp time, defined as the time between the application of the command voltage and the light stimulus, was varied. A command voltage function, programmed by the data acquisition system, was applied and the Ar III signal, the membrane current and the membrane voltage were recorded with and without a background light stimulus. Finally the bathing medium was changed from physiological saline to saline where 50% of the NaCl was replaced by LiCl and the cell was stimulated with a flash every minute.

The Ar III signals were digitally filtered with a 10th order low-pass Butterworth filter at 15 Hz. In Fig. 1 an unfiltered Ar III signal and the filtered version are laid over one another. It is seen that no information is lost due to filtering. The filtered signals were used for all further analysis.

The baseline of the Ar III signal was arbitrarily set to zero, calculated from the 400 ms before the light stimulus. The maximum of the Ar III signal was found by searching for the absolute maximum within a 1·5 s window after the light stimulus and averaging over 100 ms around this point. The time at which the absolute maximum occurred was defined as t_{max} (see Fig. 1).

In most of the Ar III signals two phases in the relaxation were clearly observed (see Fig. 3*a*). To help understand the two-phase relaxation of the Ar III signal we fitted the following function to the decay of the Ar III signal,

$$\text{Ar III}(t') = A\,e^{(-t'/\tau)} - Rt' + C. \quad (1)$$

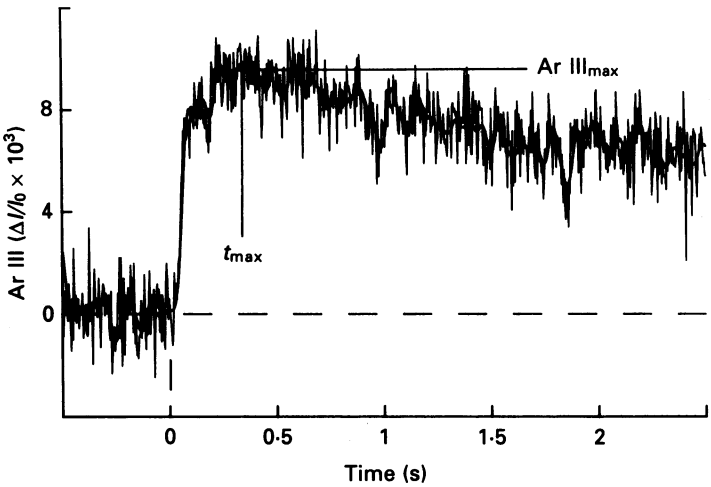

Fig. 1. Filtered and unfiltered Ar III signal. The thick line is the Ar III signal after being filtered at 15 Hz with a 10th order low-pass Butterworth filter. It is seen that no information is lost due to the filtering process. The parameters t_{max} and maximum of Ar III are shown where the computer search algorithm found them. $V_m = -60$ mV and the flash, $2{\cdot}3 \times 10^{14}$ photons cm^{-2}, occurred at time 0.

A is a scaling factor, τ is the time constant of the fast phase, R is a measure of whether the slow process is reducing $[Ca^{2+}]_i$ in the cell ($R > 0$) or increasing it ($R < 0$), and t' is defined as $t-(t_{max}+\alpha)$, where α was determined by eye and guaranteed that the fit started shortly after the observed maximum. The function in eqn (1) was fitted to the relaxation phase of the Ar III signal using a least-squares method developed by Levenberg & Marquardt (Press, Flannery, Tenkolsky & Vetterling, 1986). In Fig. 3*A* the smooth lines running through the Ar III signals are the best-fit solutions for the records. All fits were controlled by eye as well as by a χ^2 criterion (sum of the squared difference between the signal and the model function). When the signal was deemed to be unfittable because of low-frequency noise or a small signal-to-noise ratio (< 1) it was not considered. Usually not more than 5% of the records were omitted as a result of applying these criteria.

Our purpose in using the above mathematical description, is to show quantitatively that the two phases behave differently to changes in V_m. This analysis is not intended to be a complete mathematical description of the calcium regulation, but a quantitative approximation of the observed signal time course. A complete mathematical description is beyond the scope of this paper, but will be addressed in a later publication.

RESULTS

In Fig. 2 Ar III signals and receptor currents are shown under voltage clamp conditions. From this figure it is seen that the initial fast increase in internal free calcium is much slower than the receptor current; in fact the current has already reached its maximum and is starting to return to baseline as the Ar III signal

reaches its maximum. The return to baseline of the Ar III signal occurs over a long time scale and was often observed to be longer than the 10 s recording time in unclamped cells.

Light-induced $[Ca^{2+}]_i$ *increase at various* V_m *values*

In all of the seven experiments the rising phase of the Ar III signal was not significantly affected by V_m (Fig. 2). This is in contrast to the experiments of Brown

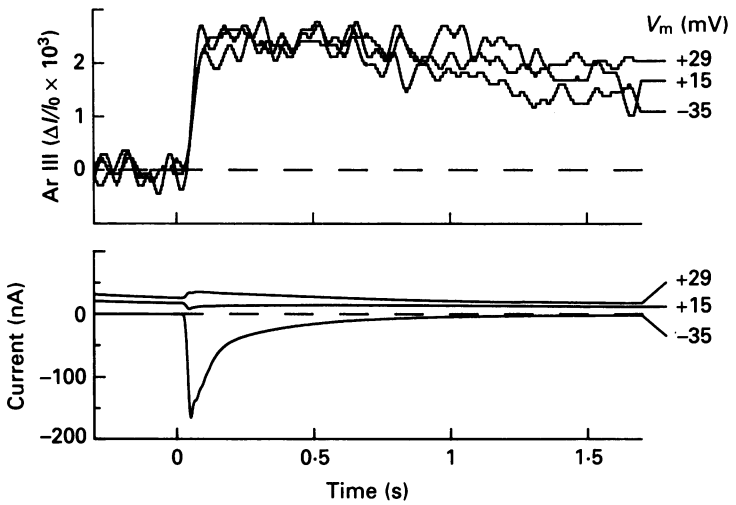

Fig. 2. Rising phase of the light-induced Ar III signal. Ar III signals elicited by a light flash ($4{\cdot}8 \times 10^{14}$ photons cm^{-2}) are shown clamped at three different V_m's. The command voltage was applied 0·5 s before the light stimulus, which occurred at time 0. Each trace is the average of three signals. The rising phase of the transient $[Ca^{2+}]_i$ increase and the maximum of the response are seen not to depend on V_m. In the lower half of the figure the receptor currents associated with the Ar III signals are plotted.

& Blinks (1974), Nagy & Stieve (1983) and Ivens & Stieve (1984) where they measured a dependence of the maximum of the calcium indicator signal on V_m. Our observation supports the hypothesis that no significant light-induced calcium conductance occurs in the plasma membrane. The discrepancy between our data and earlier data is explained later in this paper.

It is possible that due to the step in the clamp potential, voltage-activated Ca^{2+} channels were opened in the plasma membrane. In such a case we would expect to observe a Ca^{2+} conductance change within the first 200 ms after the potential step and it should depend on the size and direction of the potential step (Lisman, Fain & O'Day, 1982). This was never observed (e.g. see Fig. 3*a*). Either such channels do not exist or are not opened, or more likely a $[Ca^{2+}]_i$ change due to voltage-activated calcium channels was smaller than the resolution of our measuring system.

The measurements performed in experiments such as that shown in Fig. 2 indicated that little or no calcium originates from extracellular sources, but what

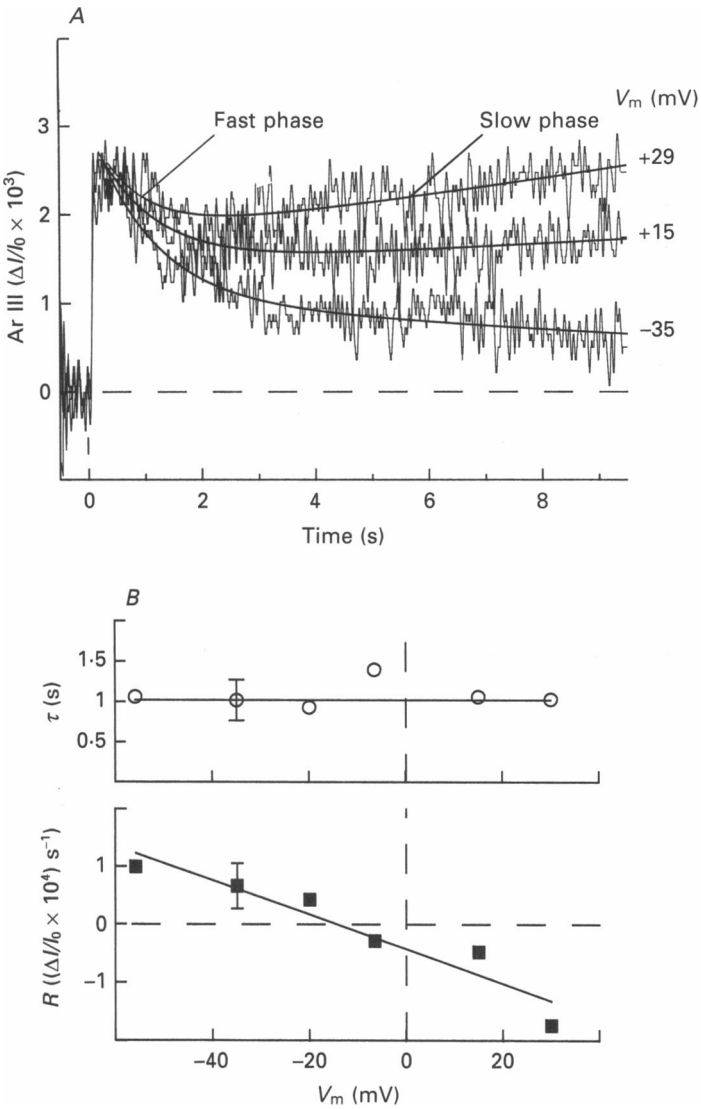

Fig. 3. Relaxation phase of the light-induced Ar III signal. *A*, the same Ar III signals are plotted as in Fig. 2. The smooth lines drawn through the curves are the best-fit solutions from eqn (1), (see Methods). The 'slow phase' is seen to depend strongly on V_m. *B*, τ and R from the fit of eqn (1) are plotted as a function of V_m. Each point is the mean of three measurements. ○, the time constant of the fast phase (τ) which is not correlated with V_m ($r = -0{\cdot}054$). The error bar is the standard deviation of all values. ■, the slope of the slow phase (R) which is negatively correlated with V_m ($r = -0{\cdot}685$). The error bar for R was calculated using χ^2 and assuming a linear fit, $(\chi^2/n-2))^{\frac{1}{2}}$.

percentage could have arrived from extracellular space unnoticed? If it is assumed that the amount of calcium entering is proportional to the driving force ($E_{Ca} - V_m$) then a change in V_m from -50 to $+30$ mV would result in a 45% reduction in the driving force, where E_{Ca} is the Nernst potential for calcium and $[Ca^{2+}]_i = 1\ \mu M$ (Levy

& Fein, 1985). If a reduction in signal size of $\frac{1}{4}$ the noise width were to go unnoticed then approximately 10% of the Ar III signal could have been caused by extracellular calcium, assuming a signal-to-noise ratio of 6:1.

In contrast to the V_m-independent rising phase of the Ar III signal, the signal after its maximum is strongly dependent on V_m (Fig. 3*A*). In six of seven experiments two phases were identified in the relaxation of the Ar III signal. Multiple phases in the calcium indicator signal were also observed by O'Day & Keller (1989). By depolarizing the plasma membrane the slow phase is reversed, that is it causes an increase in the $[Ca^{2+}]_i$. This is seen in Fig. 3*A* ($V_m = +29$ mV) as an increase in the Ar III signal long after the light stimulus is over (about 3 s afterwards). The observed characteristics of the process responsible for the slow phase behave in a manner expected of the electrogenic Na^+–Ca^{2+} exchanger described by O'Day & Keller (1989).

To quantify these observations eqn (1) was fitted to the relaxation of the Ar III signal. Figure 3*B* shows a plot of τ and R *versus* V_m, obtained from the fit of eqn (1) to the Ar III signals for the experiment pictured in Fig. 3*A*. In the six experiments in which the two phases could be identified the correlation coefficients between τ and V_m, and R and V_m were calculated. For τ they varied widely from cell to cell, from +0·388 to −0·492, but not for R, where they ranged between −0·540 and −0·921. From the observation that τ correlated weakly with V_m, sometimes positively and sometimes negatively, we conclude that changes in the process associated with τ are not dependent on V_m. However, we conclude that the process responsible for R strongly depends on V_m, since it always correlates negatively and fairly strongly with V_m.

The V_m at which the slow phase reverses direction can be derived from the fitted parameters, since at this point the slope, R, changes sign. The values for the reversal potential of slow phase vary widely, from −10 to +35 mV.

It is worth noting that the goodness of fit, P, for the mathematical description used, is approximately the same for all records from the same cell regardless of V_m. P is defined as the incomplete Γ-function,

$$P = \Gamma[\tfrac{1}{2}(n-\beta), \tfrac{1}{2}\chi^2], \tag{2}$$

where n is the number of points fitted and β is the number of fitted parameters. For a single exponential P became increasingly bad the more positive the value of V_m. In the experiment shown in Fig. 3 the P values are, for $V_m = -35$ mV, $P = 0{\cdot}987$ for eqn (1) and $P = 0{\cdot}031$ for a single exponential, and for $V_m = +15$ mV, $P = 0{\cdot}936$ for eqn (1) and $P = 2{\cdot}7 \times 10^{-9}$ for a single exponential. This result shows that the observed relaxation cannot be explained by a single exponential process.

V_m-induced change in $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$ in the ventral photoreceptor depends not only on the history of light stimulation (Nagy & Stieve, 1983; Levy & Fein, 1985) but also on the clamp history. This is demonstrated in Fig. 4. The trace in Fig. 4*A* is the average of four Ar III responses to a command voltage function. In Fig. 4*B* the trace is the measured membrane voltage in response to the command voltage function. And in Fig. 4*C* the trace is the simultaneously measured membrane current. It is seen in Fig. 4*A* that

the $[Ca^{2+}]_i$ can be influenced by changing V_m. In the right-hand side of Fig. 4 the same experiment is shown but this time the cell was additionally stimulated by a background light for 30 s. The command voltage stimuli were given every 120 s and between the stimuli the cell was either clamped at about the resting potential or unclamped. Four cells were tested.

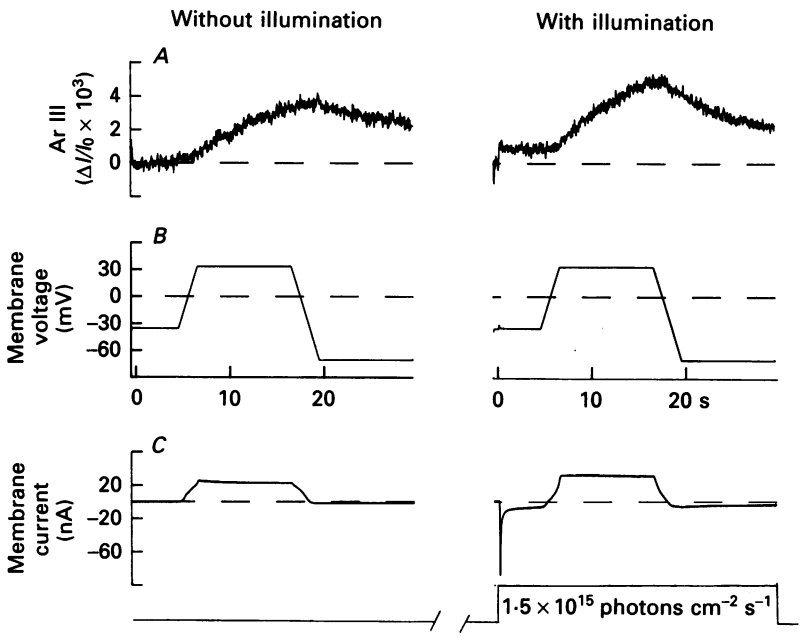

Fig. 4. Change in $[Ca^{2+}]_i$ due to altering V_m. *A*, average of four Ar III signals in response to a command voltage function. *B*, measured membrane voltage in response to the command voltage function. The associated membrane current is shown in *C*. The left-hand side is without illumination while the right-hand side is with illumination ($1{\cdot}5 \times 10^{15}$ photons $cm^{-2}\ s^{-1}$) for 30 s. The clamp quality is demonstrated in the first 5 s of the membrane voltage trace on the right-hand side. The increase and decrease in $[Ca^{2+}]_i$ is observed to be counter to the net current flow.

The results shown in Fig. 4 would be anticipated if an electrogenic Na^+–Ca^{2+} exchanger is the controlling mechanism for the resting $[Ca^{2+}]_i$ level. The data then demand a stoichiometric ratio of $Na^+:Ca^{2+} > 2$, if no other ions are involved, since $[Ca^{2+}]_i$ increases in response to a depolarization. For vertebrates such ratios have been reported: $Na^+:Ca^{2+} = 2{\cdot}7:1$ (Hodgkin *et al.* 1987). The change in $[Ca^{2+}]_i$ measured in response to a change in V_m is that qualitatively predicted by the following equation for an electrogenic exchanger:

$$[Ca^{2+}]_o/[Ca^{2+}]_i = ([Na^+]_o/[Na^+]_i)^3\, e^{-(FV_m/RT)}, \quad (3)$$

where F is the Faraday constant, R the gas constant and T the absolute temperature (Baker, 1972). In eqn (3) we have assumed a stoichiometric ratio of $Na^+:Ca^{2+} = 3:1$. Fein & Tsacopoulos (1988) measured in *Limulus* ventral photoreceptors a 10 mM $[Na^+]_i$ in the dark and a maximal transient change in $[Na^+]_i$ due to illumination of

12 mM. Assuming that during prolonged illumination the plateau value for $[Na^+]_i$ was about 11 mM in the photoreceptor, then eqn (3) predicts a 25% greater change in $[Ca^{2+}]_i$, in response to a 70 mV change in V_m, than without illumination. Such a change in $[Na^+]_i$ translates into a 2·4 mV change in the Nernst potential for Na^+, but does not necessarily imply a 2·4 mV change in the reversal potential (Fain & Lisman, 1981). This explains the observation, in Fig. 4, that the Ar III signal with a background light (right side) was about 20% larger than in the dark (left side).

It is unlikely that these observations can be explained with a Ca^{2+} conductance. An increase in $[Ca^{2+}]_i$ was observed when the cell was clamped at depolarizing potentials and a reduction was observed at hyperpolarizing potentials. This was also observed when the cell was hyperpolarized first. No activating voltage step size was observed. Rather for smaller steps, the signal was observed to be smaller. Finally the time scale of the change in $[Ca^{2+}]_i$ does not match that of other voltage-activated calcium conductances (Lisman *et al.* 1982).

Effect of V_m history on light-induced $[Ca^{2+}]_i$ increase

From the above experiments it is possible to explain the difference between our observation that the maximum of the Ar III signal was not dependent on V_m and the dependence seen by Brown & Blinks (1974), Nagy & Stieve (1983) and Ivens & Stieve (1984). Normally when investigating the characteristics of the cell under voltage clamp conditions the command voltage is applied 2–4 s before giving a light stimulus, enough time for the voltage-activated current to reach a plateau (Lisman *et al.* 1982; O'Day *et al.* 1982). When the cell is clamped at potentials other than the resting potential the equilibrium of the electrogenic exchanger is disturbed. In order to return to equilibrium the exchanger alters the $[Ca^{2+}]_i$ (Fig. 4), affecting the adaptation state of the cell. A similar result was found by O'Day *et al.* (1982) who showed that depolarizing voltage pulses desensitize the cell to light. Light adaptation has the effect of reducing the calcium signal (Maaz & Stieve, 1980; Nagy & Stieve, 1983). The longer the cell is clamped at a more positive potential than the resting potential, the more light adapted the cell becomes. We applied the command voltage 0·5 s before the light stimulus, thus avoiding this adapting effect, in contrast to the above mentioned authors. In their experiments, a longer pre-stimulus clamp time lead to an apparent dependence of the calcium indicator signal on V_m.

Direct experimental evidence supporting the above explanation is shown in Fig. 5. Four cells clamped either at −40 mV or at +22 mV, were stimulated every 60 s by a light flash. The light stimulus was given at various times after application of the command voltage. Figure 5 demonstrates that the maximum of the Ar III signal is reduced for the positive V_m, dependent on the pre-stimulus clamp time, while the maximum of the Ar III signal remains unaltered when V_m is held near the resting potential. For the four cells studied the correlation coefficients for the maximum of the Ar III signal with the pre-stimulus clamp time were between +0·034 and −0·079 for $V_m = -40$ mV and between −0·566 and −0·628 for $V_m = +22$ mV.

At most a 30% reduction of the maximum of the Ar III signal clamped at $V_m = +22$ mV over that clamped at −40 mV was observed, for pre-stimulus clamp times of up to 5 s. This is consistent with the values given by Nagy & Stieve (1983) and Ivens & Stieve (1984). Brown & Blinks (1974) indicate a 50% reduction. This

could be understood if V_m was not reduced or the clamp not turned off in the interval between measurements, as is the procedure described by Millecchia & Mauro (1969). A decrease of up to 50% was observed if V_m was held at positive values for more than 60 s (S. Benner, personal communication).

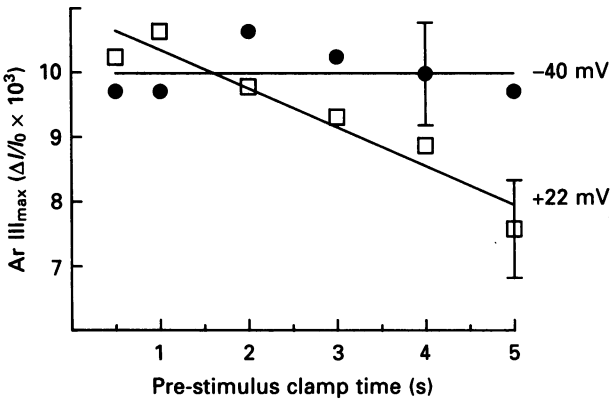

Fig. 5. Effect of V_m history on the Ar III signal. A light flash ($4{\cdot}8 \times 10^{14}$ photons cm^{-2}) was applied every 60 s and the pre-stimulus clamp time varied, while clamping the cell alternately at $V_m = -40$ or $+22$ mV. The maximum of the Ar III signal *versus* the pre-stimulus clamp time is plotted for the two V_m's. At $V_m = -40$ mV (●) the Ar III is not correlated with the pre-stimulus clamp time ($r = 0{\cdot}034$). At $V_m = +22$ mV (□) the Ar III signal is moderately correlated with the pre-stimulus clamp time ($r = -0{\cdot}580$). Each point is the mean of four measurements and the error bars are the standard deviation of all the measurements made at $V_m = -40$ mV. This experiment shows that the size of the calcium indicator signal depends on the clamp history of the cell.

Ivens & Stieve (1984) reported that the slope of the maximum of the Ar III signal *versus* V_m is steeper in low-calcium saline (250 μM) than in physiological saline. They normalized the maximum of the clamped Ar III signal by dividing it by the maximum of unclamped Ar III signals, taken alternately with clamped ones. Their normalization procedure is equivalent to dividing the actual slope by the maximum of the unclamped Ar III signal. Since the size of the Ar III signal is drastically reduced in low-calcium saline (Maaz & Stieve, 1980), their observations are explained as a normalization artifact.

Saline with 50% NaCl, 50% LiCl

Three further experiments were undertaken to determine whether the slow phase, R in eqn (1), is due to the Na^+–Ca^{2+} exchanger. Fifty per cent of the external NaCl was replaced by LiCl. Li^+ is known not to be taken up by the exchanger in vertebrate rod photoreceptors (Yau & Nakatani, 1984) and probably not in *Limulus* either (Lisman & Brown, 1972; O'Day & Keller, 1989). In Fig. 6, τ and R, calculated from Ar III signals in response to a light flash given every 60 s, are plotted as a function of the time in the 50% LiCl, 50% NaCl saline. The time constant τ was unaffected by the change in saline, in contrast to R which was reduced from 6·6 to 2·0 ($\Delta I/I_0 \times 10^4$ s^{-1}). This shows that a reduction in the Na^+ gradient results in a reduced

ability of the cell to extrude Ca^{2+}, further supporting the idea that the process responsible for the slow phase is a Na^+–Ca^{2+} exchanger in the plasma membrane. Similar observations were reported by Rüsing & Stieve (1989).

DISCUSSION

We have been able to show that the maximum of the Ar III signal is not dependent on V_m (Fig. 2). We interpret this to mean that no measurable portion of the observed

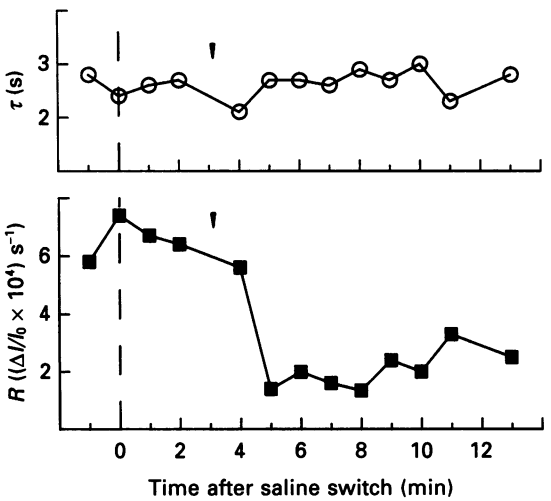

Fig. 6. Transition from physiological to 50% NaCl, 50% LiCl saline. The unclamped cell was exposed to a flash ($4{\cdot}8 \times 10^{14}$ photons cm^{-2}) every 60 s. Time 0 marks the point where the physiological saline reservoir was switched to 50% NaCl, 50% LiCl saline reservoir. The time constant τ, the fast phase of the relaxation of the Ar III signal and the slope of the slow phase, R, (see eqn (1)) were calculated for each Ar III signal and plotted against the time in the 50% NaCl, 50% LiCl saline. The arrows indicate 90% exchange of the saline solutions in the experimental chamber. Only the slope of the slow phase is seen to be affected by the reduced Na^+ gradient.

calcium increase in the cytosol comes through light-activated channels in the plasma membrane. In Fig. 3 it is shown that the relaxation of the Ar III signal is accomplished by two processes. The slow process is dependent on V_m and on $[Na^+]_o$ while the fast process is nearly unaffected by changes in these parameters (See Figs 3*A* and 6). These processes are interpreted as an electrogenic Na^+–Ca^{2+} exchanger in the plasma membrane (O'Day & Keller, 1989) and a pump mechanism in the submicrovillar cisterna of the smooth endoplasmic reticulum which removes calcium from the cytosol (Walz & Fein, 1983).

The existence of an electrogenic calcium regulation in the plasma membrane means that by changing V_m the $[Ca^{2+}]_i$ will be influenced. This was demonstrated; see Fig. 4. The way in which the calcium level is influenced by V_m is consistent with an electrogenic Na^+–Ca^{2+} exchanger having a stoichiometry of 3:1 (see eqn (6)). By changing the Na^+ and Ca^{2+} gradients across the plasma membrane it is possible to

affect the operation of the electrogenic Na^+–Ca^{2+} exchanger. A change in the ion gradients is easily accomplished by stimulating the cell with a continuous background light. Such experiments were performed and are consistent with the idea of an electrogenic Na^+–Ca^{2+} exchanger (Fig. 4). These data also indicate that the

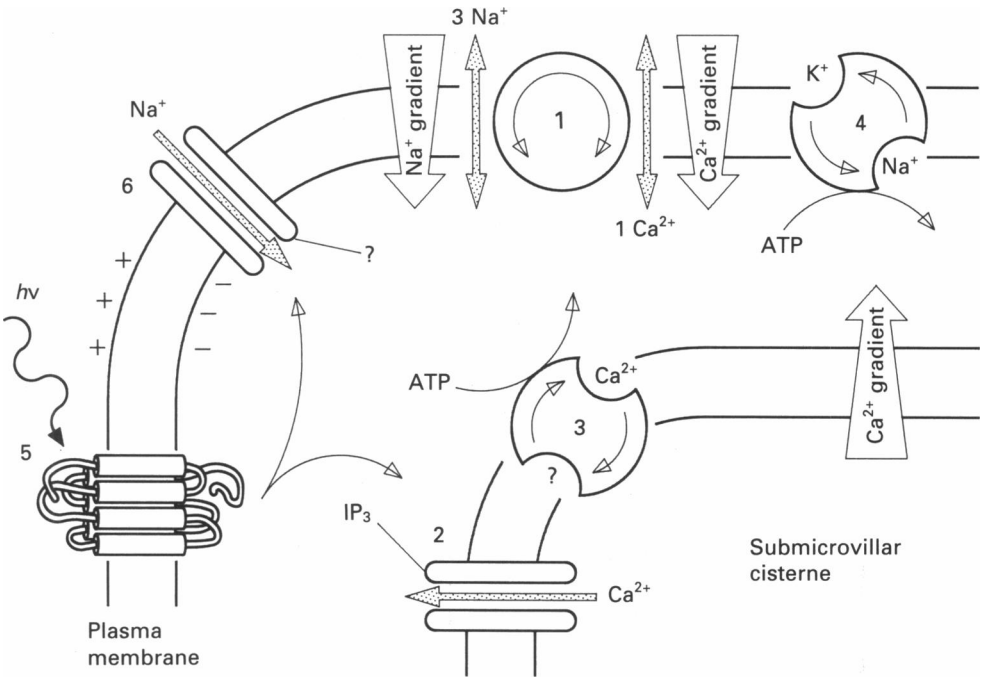

Fig. 7. Calcium regulation model. Number 1 is the electrogenic Na^+–Ca^{2+} exchanger. The exchanger attempts to create an equilibrium between the Na^+ gradient, the Ca^{2+} gradient and V_m and is primarily responsible for the long-term $[Ca^{2+}]_i$. Number 2 is an IP_3-dependent Ca^{2+} channel in the submicrovillar cisterne (SMC) activated by light (Fein & Payne, 1989). This calcium pathway is responsible for the transient increase in $[Ca^{2+}]_i$ induced by light. Number 3 is an ATP-dependent pump in the SMC responsible for refilling it (Walz & Fein, 1983). Number 4 is an electrogenic Na^+–K^+-ATPase in the plasma membrane (Brown & Lisman, 1972). Numbers 5 and 6, rhodopsin and a light-activated sodium channel, represent the process responsible for the light-induced receptor current across the plasma membrane.

exchanger is the predominant controller of $[Ca^{2+}]_i$ in the dark. If it were not, we would expect that during a change in V_m the predominant control mechanism would hold $[Ca^{2+}]_i$ to a constant level in the dark; this was not observed (see Fig. 4).

A model, which already partially exists in the literature (see Fein & Payne, 1989), is postulated for the calcium regulation in *Limulus* ventral photoreceptor. The model is shown schematically in Fig. 7. The minimal number of components required to explain the data are symbolically drawn and numbered. To our knowledge no results have been published which are inconsistent with this model.

The exchanger will always attempt to create an equilibrium between the Ca^{2+} gradient, the Na^+ gradient and V_m. Once in equilibrium, any perturbation of these

parameters will cause the exchanger to run in one direction or the other in order to re-establish equilibrium. It is theoretically possible that the exchanger is primarily responsible for controlling the long-term $[Ca^{2+}]_i$. In the dark the resting potential and the Na^+ gradient, maintained by a Na^+–K^+-ATPase (No. 4 in Fig. 7; see Brown & Lisman, 1972; Fain & Lisman, 1981), remain relatively constant. Using eqn (3), $[Ca^{2+}]_i$ is calculated to be 0·6 μM, with the reasonable assumptions that $[Na^+]_i$ = 10 mM (Fein & Tsacopoulos, 1988) and $V_m = -40$ mV. This value matches well with those measured by Levy & Fein (1985) and O'Day & Keller (1989). Some evidence exists that the exchanger in bovine rods also depends on K^+ (Schnetkamp, Basu & Szerencsei, 1989; also see Baker, 1972). This seems unlikely to be the case in *Limulus* since O'Day & Keller (1989) showed that the exchanger runs both forwards and backwards in saline containing no K^+. With the present understanding of the exchanger the interpretation of many earlier studies is made easier, such as the results obtained by injecting Na^+, Li^+ and Ca^{2+} into ventral photoreceptors (Lisman & Brown, 1972; Fein & Charlton, 1977), voltage step experiments (O'Day, Lisman & Goldring, 1982) and voltage clamp experiments with simultaneous $[Ca^{2+}]_i$ monitoring (Brown & Blinks, 1974; Nagy & Stieve, 1983; Ivens & Stieve, 1984).

We also show that the internal calcium increase does not come from a light-induced Ca^{2+} conductance increase in the plasma membrane. This leads us to agree that a light-activated calcium channels (via inositol 1,4,5-trisphosphate, IP_3) exists in the submicrovillar cisterna (No. 2 in Fig. 7) which is responsible for the increase in $[Ca^{2+}]_i$. The seminal evidence for this calcium pathway comes from the work of a number of groups or investigators who showed that this channel is activated by IP_3 (Brown & Rubin, 1984; Fein *et al.* 1984; Fein & Payne, 1989).

The existence of an ATP-dependent calcium pump in the submicrovillar cisterna (No. 3 in Fig. 7; Walz & Fein, 1983) explains why we observed another process besides the electrogenic Na^+–Ca^{2+} exchanger in the relaxation of the Ar III signal. Regulation of the calcium level by the calcium pump in the submicrovillar cisterna and the exchanger in the plasma membrane explains the Bolsover & Brown (1985) observe a light stimulus-dependent emptying of the submicrovillar cisterna (also see Maaz & Stieve, 1980; Levy & Fein, 1985). It also explains an observation of Levy & Fein (1985) that after a bright flash of light the $[Ca^{2+}]_i$ transiently decreases below the resting level. This observation can be explained by the model if the submicrovillar cisterna pump is able to reduce the $[Ca^{2+}]_i$ below the equilibrium level for the exchanger. The exchanger would then run in reverse, that is increase the internal calcium, in order to bring itself back into equilibrium. This would continue until the gradient across the submicrovillar cisterna membrane is re-established.

A couple of other possibilities could contribute to the calcium regulation in the *Limulus* ventral photoreceptor which are important in other systems. One possibility is a significant leakage of calcium across the plasma membrane, as is the case in muscle cells (Campbell, 1985). A second possibility is the existence of a Ca^{2+}-ATP pump in the plasma membrane as in erythrocytes (Baker, 1972). No data exists, to our knowledge, which would force us to assume that either of these mechanisms contributes significantly to calcium regulation in *Limulus*. A voltage-activated calcium conductance as proposed by Lisman *et al.* (1982) does not appear to contribute significantly under our experimental conditions, as already discussed. In

the well-studied squid axon system Ca^{2+} efflux can be explained by an electrogenic Na^+–Ca^{2+} exchanger and an ATP-dependent Na^+ pump (Requena & Mullins, 1979), as proposed in our model.

Apparently an increase in $[Ca^{2+}]_o$ causes an increase in the Ca^{2+} gradient across the submicrovillar cisterna membrane. This expresses itself as an increase in the light-activated calcium indicator signal (Brown & Blinks, 1974; A. Deckert, unpublished). On the other hand, if the cell experiences a strong adaptation due to the increase in $[Ca^{2+}]_o$, the response to a light stimulus will be reduced, including the calcium indicator signal, as reported by Ivens & Stieve (1984).

After a bright illumination the time course of calcium-dependent dark adaptation, the initial fast recovery, (Stieve & Pflaum, 1978; Levy & Fein, 1985; Claßen-Link & Stieve, 1986) matches very well with the relaxation time of the Ar III signal of 6–20 s (90% recovery). Values in the literature vary between 3–20 s (Fein & DeVoe, 1973; Nagy & Stieve, 1984; Claßen-Linke & Stieve, 1986).

There seem to be important differences in the regulation of $[Ca^{2+}]_i$ in microvillar photoreceptors of different groups of invertebrates. For example, in photoreceptors of the barnacle *Balanus* and the fly *Calliphora* it has been shown that light-activated calcium currents exist across the plasma membrane (Brown, Hagiwara, Koike & Meech, 1970; Brown & Blinks, 1974; P. Hochstrate, personal communication). In the ommatidia of the crayfish *Orconectus* the exchange of calcium with the exterior medium appears to be independent of the light stimulus programme (Becker, Stieve & Wendel, 1988).

We wish to thank Dr K. Nagy for careful reading and very helpful criticism of this manuscript in all stages of its development. We are also grateful to Susanne Benner for many enlightening discussions, to Dr J. Schnakenburg for his helpful discussions related to the model presented, to Helmut Reuss for invaluable help in programming and to H. T. Hennig for his help with Fig. 7. The author A. Deckert would also like to express his thanks to Dr U. Becker and Dr K. Nagy for many hours of patient help in the experimental phase of this work.

REFERENCES

Baker, P. F. (1972). Transport and metabolism of calcium ions in nerve. *Progress in Biophysics and Molecular Biology* **24**, 177–223.

Becker, U. W., Stieve, H. & Wendel E. (1988). Is desensitization of crayfish retinula due to calcium depletion independent of stimulation? *16th Göttingen Neurobiology Conference*. Abstract in *Sense Organs, Interfaces between Environment and Behavior*, ed. Elsner, N. & Barth, F., p. 207. Georg Thieme Verlag, Stuttgart, New York.

Blinks, J. R., Wier, W. G., Hess, P. & Prendergast, F. G. (1982). Measurement of Ca^{2+} concentrations in living cells. *Progress in Biophysics and Molecular Biology* **40**, 1–114.

Bolsover, S. R. & Brown, J. E. (1985). Calcium ion, an intracellular messenger of light adaptation, also participates in excitation of *Limulus* photoreceptors. *Journal of Physiology* **364**, 381–393.

Brown, H. M., Hagiwara, S., Koike, H. & Meech, R. M. (1970). Membrane properties of a barnacle photoreceptor examined by the voltage clamp technique. *Journal of Physiology* **208**, 385–413.

Brown, J. E. & Blinks, J. R. (1974). Changes in intracellular free calcium concentration during illumination of invertebrate photoreceptors. *Journal of General Physiology* **64**, 643–665.

Brown, J. E., Brown, P. K. & Pinto, L. H. (1977). Detection of light-induced changes of intracellular ionized calcium concentration in *Limulus* ventral photoreceptors using Arsenazo III. *Journal of Physiology* **267**, 299–320.

BROWN, J. E. & LISMAN, J. E. (1972). An electrogenic sodium pump in *Limulus* ventral photoreceptor cells. *Journal of General Physiology* **59**, 720–733.

BROWN, J. E. & MOTE, M. I. (1974). Ionic dependence of reversal voltage of the light response in *Limulus* ventral photoreceptors. *Journal of General Physiology* **63**, 337–350.

BROWN, J. E. & RUBIN, L. J. (1984). A direct demonstration that inositol-triphosphate induces an increase in intracellular calcium in *Limulus* ventral photoreceptors. *Biochemical and Biophysical Research Communications* **125**, 1137–1142.

CAMPBELL, A. (1985). Intracellular calcium, its universal role as regulator. John Wiley and Sons Ltd, Chichester, New York, Brisbane, Toronto, Singapore.

CLAßEN-LINKE, I. & STIEVE, H. (1986). The sensitivity of the ventral nerve photoreceptor of *Limulus* recovers after light adaptation in two phases of dark adaptation. *Zeitschrift für Naturforschung* **41c**, 657–667.

FAIN, G. L. & LISMAN, J. E. (1981). Membrane conductances of photoreceptors. *Progress in Biophysics and Molecular Biology* **37**, 91–147.

FEIN, A. & CHARLTON, J. S. (1977). A quantitative comparison of the effects of intracellular calcium injection and light adaptation on the photoresponse of *Limulus* ventral photoreceptors. *Journal of General Physiology* **70**, 591–600.

FEIN, A. & DEVOE, R. D. (1973). Adaptation in the ventral eye of *Limulus* is functionally independent of the photochemical cycle, membrane potential, and membrane resistance. *Journal of General Physiology* **61**, 273–289.

FEIN, A. & PAYNE, R. (1989). Phototransduction in *Limulus* ventral photoreceptors: roles of calcium and inositol triphosphate. In *Facets of Vision*, chap. 9, ed. STAVENGA, D. & HARDIE, R. Springer-Verlag, Berlin, Heidelberg.

FEIN, A., PAYNE, R., CORSON, D. W., BERRIDGE, M. J. & IRVINE, R. F. (1984). Photoreceptor excitation and adaptation by inositol 1,4,5-triphosphate. *Nature* **311**, 157–160.

FEIN, A. & TSACOPOULOS, M. (1988). Light-induced oxygen consumption in *Limulus* ventral photoreceptors does not result from a rise in the intracellular sodium concentration. *Journal of General Physiology* **91**, 515–527.

HODGKIN, A. L., MCNAUGHTON, P. A. & NUNN, B. J. (1987). Measurement of sodium–calcium exchange in salamander rods. *Journal of Physiology* **391**, 347–370.

IVENS, I. & STIEVE, H. (1984). Influence of the membrane potential on the intracellular light induced Ca^{2+}-concentration change of the *Limulus* ventral photoreceptor monitored by Arsenazo III under voltage clamp conditions. *Zeitschrift für Naturforschung* **39c**, 986–992.

LEVY, S. & FEIN, A. (1985). Relationship between light sensitivity and intracellular free Ca concentration in *Limulus* ventral photoreceptors. *Journal of General Physiology* **85**, 805–841.

LISMAN, J. E. & BROWN, J. E. (1972). The effects of intracellular iontophoretic infection of calcium and sodium ions on the light response of *Limulus* ventral photoreceptors. *Journal of General Physiology* **59**, 701–719.

LISMAN, J. E., FAIN, G. L. & O'DAY, P. M. (1982). Voltage-dependent conductance in *Limulus* ventral photoreceptors. *Journal of General Physiology* **79**, 187–209.

MAAZ, G. & STIEVE, H. (1980). The correlation of the receptor potential with the light induced transient increase in intracellular calcium-concentration measured by absorption change of Arsenazo III injected into *Limulus* ventral nerve photoreceptor cell. *Biophysics of Structure and Mechanism* **6**, 191–208.

MILLECCHIA, R. & MAURO, A. (1969). The ventral photoreceptor cells of *Limulus*: III. A voltage clamp study. *Journal of General Physiology* **54(3)**, 331–351.

NAGY, K. & STIEVE, H. (1983). Change in intracellular calcium ion concentration in the course of dark adaptation measured by Arsenazo III in the *Limulus* photoreceptor. *Biophysics of Structure and Mechanism* **9**, 207–223.

O'DAY, P. M. & KELLER, M. P. (1989). Evidence for electrogenic Na^+/Ca^{2+}-exchange in *Limulus* ventral photoreceptors. *Journal of General Physiology* **93**, 473–492.

O'DAY, P. M., LISMAN, J. E. & GOLDRING, M. (1982). Functional significance of voltage-dependent conductances in *Limulus* ventral photoreceptors. *Journal of General Physiology* **79**, 211–232.

PRESS, W. H., FLANNERY, B. P., TEUKOLSKY, S. A. & VETTERLING, W. T. (1986). *Numerical Recipes. The Art of Scientific Computing*, chap. 14. Cambridge University Press, Cambridge.

REQUENA, J. & MULLINS, L. (1979). Calcium movement in nerve fibers. *Quarterly Reviews in Biophysics* **12**, 371–460.

RÜSING, G. & STIEVE, H. (1989). Investigations on the calcium sodium antiport in the ventral nerve photoreceptor cell of *Limulus Polyphemus*. *Deutsche Neurobiologentagung Göttingen*. Abstract in *Dynamics and Plasticity in Neuronal Systems*, ed. ELSNER, N. & SINGER, W., p. 92. Georg Thieme Verlag, Stuttgart, New York.

SCHNETKAMP, P. P. M., BASU, D. K. & SZERENCSEI, R. T. (1989). Na^+–Ca^{2+} exchange in bovine rod outer segments requires and transports K^+. *American Journal of Physiology* **257**, C153–157.

STIEVE, H. & PFLAUM, M. (1978). The response height versus stimulus intensity curve of the ventral nerve photoreceptor of *Limulus* depending on adaptation and external calcium concentration. *Vision Research* **18**, 747–749.

WALZ, B. & FEIN, A. (1983). Evidence for calcium-sequestering smooth ER in *Limulus* ventral photoreceptors. *Investigations in Ophthalmology and Vision Science* **24**, suppl., 281.

YAU, K. W. & NAKATANI, K. (1984). Electrogenic Na^+–Ca^{2+} exchange in retinal rod outer segment. *Nature* **311**, 661–663.