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Changes in Intracellular Calcium Ion Concentration, in the Course of Dark Adaptation Measured by Arsenazo III in the *Limulus* Photoreceptor

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Abstract. In experiments on the ventral nerve photoreceptor cells in *Limulus* the transient absorption changes of the intracellularly pressure-injected calcium indicator dye, Arsenazo III which were evoked by 10 ms intense light flashes were recorded at 645 nm together with the corresponding receptor potential. The Arsenazo signal provided a monitor of the intracellular calcium ion concentration during different experimental conditions.

1) The time between the peak of the Arsenazo signal and that of the receptor potential was shorter (ca. 50 ms) when small amounts of Arsenzo where injected into the cell. This indicates that there may be little or no delay between membrane voltage signal and increase in intracellular calcium.

2) Results obtained under voltage clamp conditions show that the Arsenazo signal depends on the membrane potential. Plotting the amplitude of the Arsenazo signal as function of the time integral of the light induced membrane current signal (which corresponds to the bulk of tranported charge), results in a linear proportionality. This may indicate that the light induced increase in intracellular calcium is due to a constant fraction of the ion current underlying the receptor current.

3) During the time course of dark adaptation following a light adapting flash, two phases of recovery of the amplitude h_T, of the time integral of the receptor potential and of the amplitude A_T of the Arsenazo signal evoked by a constant test flash can be seen (Fig. 5). The first phase correlates with the decrease of the prestimulus level A_P of the intracellular calcium ion concentration which had been raised due to the light adaptation. The recovery of the amplitude of the Arsenazo signal and the time integral of the receptor potential appear to be similar both in time course and in relative

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amplitude of the two phases whereas the recovery of the height h_T of the receptor potential seems to be attenuated in the second phase.

This two-phase recovery indicates the operation of two mechanisms regulating the sensitivity of the photoreceptor. Only the first phase seems to be strongly correlated with the decline of the intracellular calcium ion concentration.

Key words: *Limulus* ventral nerve photoreceptor – Intracellular calcium concentration change – Arsenazo III – Dark adaptation – Voltage clamp

Introduction

It has been known for some years, that the intracellular free calcium plays an important role in the regulation of the state of light adaptation of invertebrate photoreceptors, but the precise mechanism is not yet clear (for review see: Stieve 1981).

Lisman and Brown (1972, 1975) and Fein and Lisman (1975), showed that injection of calcium reduce the amplitude of the photoresponse in *Limulus* photoreceptors. Brown and Blinks (1974), using the calcium sensitive photoprotein aequorin, have demonstrated that the light stimulus elicits a transient increase in intracellular calcium concentration in the *Limulus* ventral eye. From voltage-clamp experiments they inferred that the increase in $[Ca^{2+}]_i$ is partly due to the influx of calcium ions but they also supposed that there is a release of calcium from intracellular stores because the increase in intracellular calcium also occurs in calcium free sea water.

Brown et al. (1977) using the calcium indicator dye, Arsenazo III, estimated the intracellular Ca^{2+}-ion concentration to be in the range of 10^{-4} mol/l in a strongly light adapted cell.

Correlation between the receptor potential (ReP) and the change of the extracellular Ca^{2+}-concentration was studied by Maaz and Stieve (1980). They found that the transient increase of $[Ca^{2+}]_i$ evoked by a brief light flash is proportional to the time integral of the voltage displacement of the light response (i.e., receptor potential) – and therefore also proportional to the half-time of decay t_2 of the ReP – but not proportional to the height of the ReP[1]). Maaz et al. (1981) showed that the ReP consists of at least two components one of which is mainly responsible for the $[Ca^{2+}]_i$ increase (Maaz and Stieve 1980).

The work presented here studies the time course of dark adaptation in *Limulus* ventral nerve photoreceptor by comparing ReP and the changes in $[Ca^{2+}]_i$ as measured with Arsenazo III.

The metallochromic indicator dye, Arsenazo III is a sensitive Ca^{2+} detector which can be used in electrophysiological studies (Brown et al. 1977; Brown and Pinto 1979). The dye forms a complex with calcium ions (with relatively short rate constant of 2.8 ms; Scarpa et al. 1978) accompanied by a change in colour thus offering a spectroscopic method to measure Ca^{2+}-concentrations. Concerning the stoichiometry of the calcium-Arsenazo complex there is no agreement in the literature. The works of Thomas (1979) and Thomas and Gorman (1978) state that the 1 : 1 Ca^{2+}-Arsenazo complex is formed only at very low dye concentration, whereas at the generally used dye concentrations (near to the millimolar range) the 1 : 2 Ca^{2+}-Arsenazo complex is formed. Other authors (Bauer 1981; Kendrick 1976; Scarpa et al. 1978) report 1 : 1 stoichiometry of the complex. There are also difficulties in quantitative analyses caused by the strong binding constant of Ca^{2+} to the Arsenazo III (10^4–10^5 M^{-1}, Ohnishi 1979). But there is agreement that it is reasonable to assume that the absorption of the dye changes linearly with the calcium concentration in the range of concentrations of Arsenazo and calcium present in the cell under our experimental conditions.

The results we present here concern the dark adaptation of *Limulus* ventral eye by simultaneously measuring the receptor potential and the absorption change of the Arsenazo III in the course of dark adaptation. After an intense, short light pulse the dark adaptation process is scanned by test flashes. Two

distinct phases of dark adaptation are observed in the time course of the recovery of the amplitudes of the receptor potential and of the light induced Arsenazo signal. The first phase of recovery coincides in time course with the decline of the prestimulus level of intracellular ionized calcium, indicating the regulating effect of calcium ions on the sensitivity during dark adaptation. In the second phase of dark adaptation, calcium probably has not the controlling influence on the regulation of the adaptation. Our voltage-clamp experiments indicate that the light induced increase in intracellular Ca^{2+}-concentration is proportional to the total amount of charge transported by the receptor current, indicating a light induced Ca^{2+}-inward current being a (probably constant) fraction of the

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[1]) The following abbreviations are used:

ReP	receptor potential (= membrane voltage signal)
ReC	receptor current (= membrane current signal under voltage clamp conditions)
PMP	prestimulus membrane potential
U_m	membrane potential
h_C, h_T	amplitude (height of the maximum) of the ReP elicited by the conditioning (light adapting) or the test flash, respectively
$h^T = \frac{h_T}{h_C}$	normalized amplitude of test flash induced ReP
A_C	amplitude of the Arsenazo signal elicited by the conditioning
A_T	or the test flash and
A_P	Arsenazo level measured just before the test flash
A_{free}	amplitude of Arsenazo signal obtained in free (unclamped) and
A_{CL}	under voltage clamp conditions
$A'_T = \frac{A_T}{A_C}; A'_{CL} = \frac{A_{CL}}{A_{\text{free}}}$	normalized values of the
$A'_P = \frac{A_P}{A_C}$	Arsenazo response
J_M	membrane current,
J_D	membrane current in the dark and
ΔJ_L	light induced membrane current amplitude under voltage clamp
t_2	half time of decay of the receptor potential
$\int U_{\text{ReP}}$	time integral of the ReP elicited by the test flash
$\int J_{\text{ReC}}$	time integral of the ReC
DA	dark adaption
t_{DA}	dark adaptation time, measured from the start of the conditioning flash to the test-flash
$t_{\max}$	the time to peak measured from the start of the stimulus for ReP, ReC, and Arsenazo signal
t_c	cycle time
VNP	ventral nerve photoreceptor

receptor current through the photoreceptor cell membrane. A brief report has already been included in a previous review (Stieve 1982).

Material and Methods

Limulus ventral nerves were dissected under dim white light and were, after desheathing, treated with 0.5% pronase for 45 s. The ventral nerve was placed on a piece of Sylgard in an experimental chamber, which was continuously perfused with physiological saline. The perfusion was interrupted for a few seconds during each voltage-clamp measurement. The composition of the saline was (mmol/l): 488 Na^+, 10 K^+, 55 Mg^{2+}, 10 Ca^{2+}, 566 Cl^-, 30 SO_4^{2-}, 2.3 HCO_3^-, 0.1 PO_4^{3-}, and 10 glucose. Microelectrodes filled with 0.5 mol/l KCl solution having a resistance of 10–15 MΩ were used. The voltage sensor electrode, containing 10–20 mmol/l Arsenazo III in 0.5 M KCl solution, was used for pressure injection of the dye. Arsenazo III was purified according to Kendrick (1976). All experiments were carried out at room temperature, which was about 20° C.

The block diagram of the setup (modified from Maaz and Stieve 1980) is shown in Fig. 1. The measuring light, having a 2×10^{-8}–10^{-7} W intensity of continuous red light (Schott interference filter, λ_{max} = 645 nm, half width 9 nm), was focused on the cell by a microscope objective (magnification 32×). The diameter of the light spot could be varied from 50 μm to 300 μm with the help of a variable diaphragm. Mostly a diameter of ca. 70 μm was used. The measuring light passed the photoreceptor cell horizontally and was movable along two axes, perpendicular to the light pathway. In this way the light spot could be aimed at the most active area of the cell.

The direction of the stimulus light was vertical. For stimulation, 10 ms white light flashes (Hg high pressure lamp, HBO 100, Osram) light intensity of 7 mW/cm^2 timed by an electromechanical shutter, were used. A microscope objective focused the stimulating light on the cell. A photomultiplier tube (EMI 9801 B), having an interference filter (λ_{max} = 645 nm, half width 8 nm) in front of its window to reduce the flash artefact, measured the transmission change of the cell with the injected dye.

Moderate amounts of the injected dye (2–3 mmol/l) caused a small change in the receptor potential (a small shoulder in the falling phase, as reported by Maaz and Stieve 1980), which became more pronounced with increasing dye concentration. Much higher dye concentration reduced the Arsenazo signal.

Different dye concentrations were used by different authors. Brown et al. (1977) report that 0.7–3.0 mmol/l dye concentration does not influence the kinetics of the receptor potential and of the

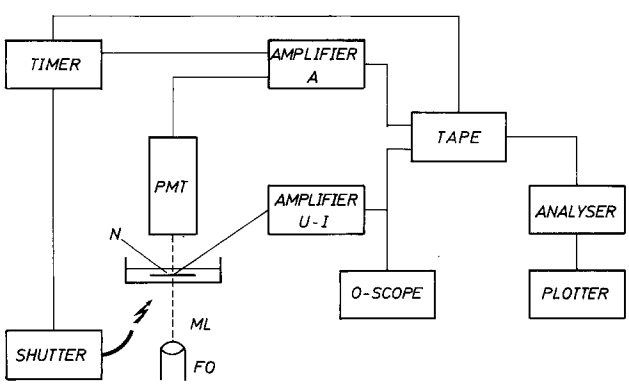


Fig. 1. Block diagram of the setup. A microscope objective (*FO*) focusses the measuring light (*ML*) on the photoreceptor cell located on the ventral nerve (*N*). A photomultiplier (*PMT*) measures the transmission change. The output signal of the multiplier is amplified (*A*) and recorded on tape simultaneously with the membrane voltage ReP or the membrane current ReC signal (*U-I*). The shutter gives the 10 ms light pulses. A digital timer ensures the periodicity and triggers each instrument

Arsenazo signal. According to Thomas (1979) an appropriate concentration of the intracellular dye is about 0.6–2.0 mmol/l. The dye concentration used by us is estimated (from the absorbance change of the cell due to the dye injection at 645 nm and from the optical pathlength) to have been between 0.7 and 2.5 mmol/l. Then the Arsenazo signal was large enough to get reasonable signal to noise ratios in most cases without averaging. Normally, the intensity change of the measuring light due to the complex formation of the dye was above 8×10^{-4}. In the experiments in which the rate of the intracellular calcium increase was of special importance (Fig. 3) a smaller quantity of dye was injected; in these cases some averaging was necessary to get reasonable records. The measuring light intensity was so low that it did not cause an essential change of the prestimulus membrane potential PMP. The depolarization was less than 4%, when the measuring light was switched on to a dark adapted cell. Before the injection of the dye into the cell we could not observe any light induced change in the transmission of the cell, i.e., the light scattering of the cell did not change measurably due to the excitation.

After the pressure injection of the Arsenazo we stimulated the photoreceptor with 10 ms flashes (repetition time 30 ms) for 15–20 min to allow for homogeneous distribution of the dye. During this preperiod the shape and the height of the Arsenazo signal changes a little, but later it stayed stable for 2–3 h. The injection of the dye causes somewhat longer receptor potentials and sometimes depolarizes the cell by a few millivolts.

In the measurements of the time course of dark adaptation, pairs of flashes were applied. A conditioning (light adapting) flash was repeated periodically after a cycle time t_c. This conditioning flash was followed by an identical test-flash after a delay time which was varied from cycle to cycle between 0.5 and 50 s. For delay times up to 10 s the cycle time was 90 s, for longer delays, cycle times of 3 min were used. The time course of the experiment and the measured characteristic records are illustrated in Fig. 2a. The program of the voltage-clamp experiments is shown in Fig. 2b. A Datalab 4000 averager and microprocessor was used to measure the time integral of the receptor potential ReP or of the receptor current, ReC.

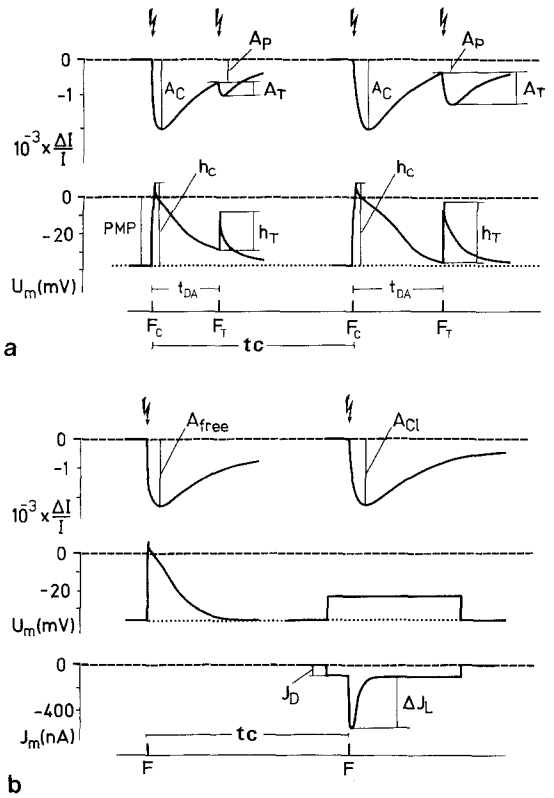


Fig. 2a and b. Stimulus regime in the experiments and the obtained data in the adaptation test (**a**) and in the voltage clamp measurements (**b**). F_c the first, conditioning (adapting) flash, F_T the second, test flash applied during a cycle (t_c). t_{DA} is the delay time between the adapting and test flash. h_C, h_T indicate the amplitudes of the receptor potential, A_C, A_T the amplitudes of the absorption signal. A_P indicates the Arsenazo level before the test flash. A_{free} shows Arsenazo response of the unclamped and A_{CL} of the clamped cell; ΔJ_L is the amplitude of the light induced membrane current ReC. J_D dark current

Results

Measurements of the Time-to-Peak of the Intracellular Ca^{2+}-Concentration Change

When we studied large photoreceptor cells we observed that there are active spots in the cell in which the transmission change of the dye is much higher than in other regions. So, 15–20 min after the injection of the dye (to allow an even distribution throughout the cell), we focussed the measuring light spot on the most active area.

It is known (Brown et al. 1977) that the kinetics of the transmission change of the injected Arsenazo III (Arsenazo signal) depend on the intracellular dye concentration. The higher the dye concentration in the cell, the slower the rise time of the Arsenazo signal. It has been shown by Maaz and Stieve (1980) that the time-to-peak of the Arsenazo signal in *Limulus* ventral nerve photoreceptor using a convenient intracellular dye concentration (ca. 3 mmol/l) for a ReP amplitude saturating stimulus intensity is about 800 ms. (Sometimes a faster component could be observed.)

Here we observed (Fig. 3) that with low intracellular dye concentration the time-to-peak varied between 90 and 140 ms (7 cells were measured). It can be seen in the same figure, that the time delay between the peak of the receptor potential and that of the Arsenazo signal is ca. 50 ms. We could not measure the latent period of the Arsenazo signal because of the flash artefact and electrical noise but it is probably in the same range as the latency of the receptor potential.

The decline of the Arsenazo signal to the prestimulus level is slower by a factor of 1.5–2.0 than the decline of the receptor potential, as already observed by Maaz and Stieve (1980) (e.g., Fig. 4b).

Time Course of the Dark Adaptation

Using a double flash technique (see Fig. 2a) we scanned the time course of the dark adaptation of photoreceptor cells (5 experiments) and measured the dependency of ReP and Arsenazo signal upon dark adaptation time (t_{DA}). Two identical 10 ms flashes were applied to the dark adapted cell with a cycle time (t_c) of 90 s or 3 min. The delay time (t_{DA}) between the two flashes was increased from cycle to cycle. So the dark adaptation of the cell was scanned by identical test flashes after different delays. Two records are shown in Fig. 4.

In Fig. 4a one can see that the level of Arsenazo transmission A_P (after 4 s) has returned to about 25% of the amplitude A_C of the Arsenazo signal evoked by the conditioning flash. At the same time the test-flash evokes only a small Arsenazo response A_T (about 25% of the dark adapted amplitude) and a receptor potential with an amplitude of ca. 60% of that of the dark adapted response (however their peak voltages are the same). Even 30 s after the light adapting flash (Fig. 4b) the Arsenazo level A_P has not yet reached the original value. From the whole set of measurements, for which Fig. 4a and b are examples, the dependence of the recovery of the light response on the time of

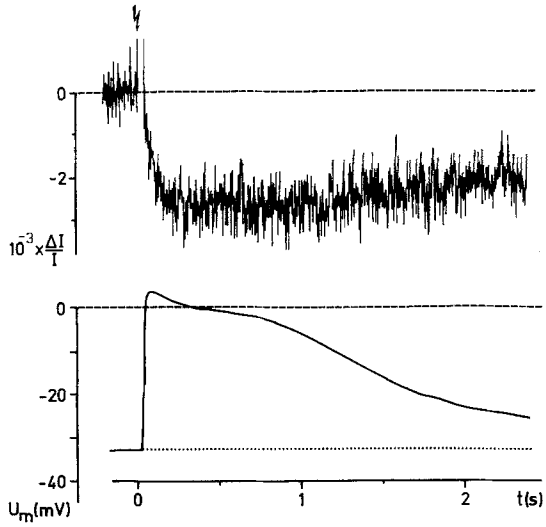
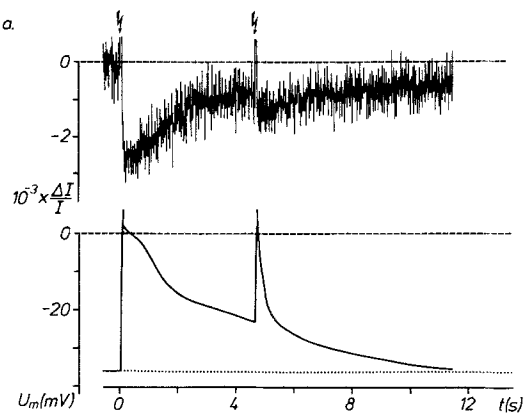


Fig. 3. Test of the rise time of the Arsenazo signal (upper curve) and the simultaneous receptor potential (lower curve). The prestimulus membrane potential was 34 mV. The record is the average of four signals. Light intensity of 7 mW/cm^2 was applied for 10 ms, which gave a saturated heigth of ReP. Repetition rate: 90 s. *Limulus* VNP



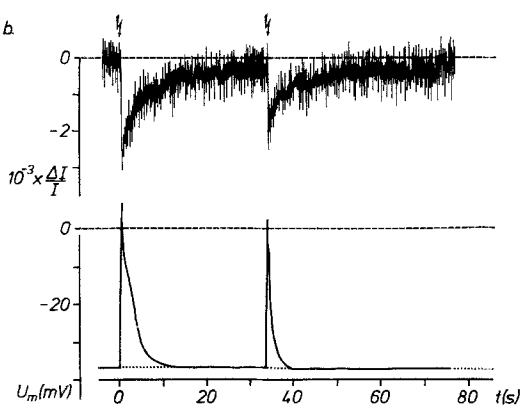


Fig. 4a and b. Test of the course of dark adaptation. Conditioning and test flash induced Arsenazo signal and ReP at two different times t_{DA} of dark adaptation: **a** 4.6 s and **b** 33.0 s. Light intensity as in Fig. 3. The stimulus program of the experiment is shown in Fig. 2. The PMP was −36 mV; curves **a** and **b** are obtained with the same cell as in Fig. 3. *Limulus* VNP

dark adaptation t_{DA} can be evaluated. In Fig. 5a and b the amplitude h'_T and time integral of the receptor potential, the height A'_T of the Arsenazo response evoked by the test flash and the prestimulus level A'_P of the Arsenazo transmission are plotted for test stimuli after different dark adaptation times t_{DA}. (These values are normalized with respect to the corresponding values of the signal elicited by the preceding light adapting flash.)

The curves in Fig. 5 show some important characteristics: The recovery of the amplitude h_T, that of the time integral of the receptor potential and that of the amplitude of the Arsenazo response (A'_T) evoked by the test flash, have two phases. The first phase of the recovery of the receptor potential reaches its maximum (which is more than 90% of the dark adapted response height h_C) within less than 10 s after the light adapting flash. Then, after a slight decline, the second phase of recovery begins. Now the time integral and the half-width of the ReP show more than 60% of their growth in the second phase.

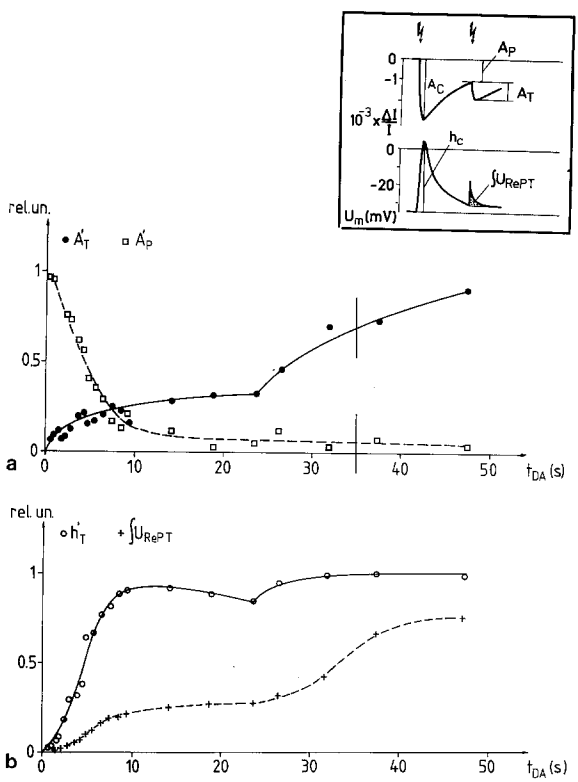


Fig. 5a and b. Change of the Arsenazo signal and the ReP amplitude during dark adaptation. **a** Test flash induced maximum A'_T (filled circles) and prestimulus level A'_P (open squares) of the Arsenazo signal as function of the delay time t_{DA}. **b** Dependence of the height h'_T and of the time integral of the test flash evoked ReP on the dark adaptation time t_{DA}. Data obtained from same experiment as presented in Fig. 4 are normalized with respect to the responses evoked by the conditioning flash (see abbreviations). Bars indicate the noise amplitudes. Inset explains the measured parameters. *Limulus* VNP

This second phase is due to the appearance of the second component C_2 of the ReP (Maaz et al. 1981), which appears as a slight shoulder in the decline of the ReP elicited by the test flash in Fig. 4b. The two components of the receptor potential are characterized in Maaz et al. 1981. The decline of the prestimulus Arsenazo level A'_P to the level before the light adapting flash appears to be an exponential decay during the first 10 s (open squares in Fig. 5a). Although the transmission A'_P has returned to as much as a quarter of the dark adapted value, after about 8 s, the test flash evokes at this time t_{DA} only a small Arsenazo response (A_T) (Fig. 4 and 5).

In Fig. 5a the first phase of the recovery of the Arsenazo response A'_T elicited by the test flash (filled circles) takes ca. 20 s to approach a plateau, which is about one-third of the response amplitude (A_C) obtained in the dark adapted cell. The second phase of A_T recovery starts at the same time as the second phase of the recovery of the ReP height h_T. The decline of the prestimulus Arsenazo level (A_P) shows a monotonic slow decrease during that time interval (open squares in Fig. 5a). Only in the second phase of the recovery does the Arsenazo response reach amplitudes (A_T) larger than 50% of the value obtained with the dark adapted cell. Under our experimental conditions full dark adaptation was not achieved in less than 60 s after the light adapting flash.

The Arsenazo response correlates well (see below and Maaz and Stieve 1980) with the time integral or the half-time of decline of the ReP; these two parameters are also sensitive characteristics of the state of dark adaptation (Stieve unpublished).

All of the six tested cells showed curves similar in character to those presented in Fig. 5. During the second phase of dark adaptation (which starts between 10 and 25 s after the light adapting falsh) there was no correlation between Arsenazo signal A'_T evoked by a test flash and A'_P, the level of the Arsenazo. In all experiments the second phase of recovery of the receptor potential started almost simultaneously (i.e., within 4 s) with the second phase of recovery of the Arsenazo signal. The plateau amplitude of the first phase of the Arsenazo response A'_T (filled circles in Fig. 5) varied from cell to cell between 0.2 and 0.5 (on the relative scale). The variation of these parameters may depend not only on the cell itself, but also on the injected amount of the Arsenazo III.

We analyzed the relationship between the Arsenazo response and the width of the receptor potential. The width of the ReP can be characterized by the decay time t_2 or by the time integral of the ReP. We plotted the maximum of the Arsenazo response A'_T as the function of t_2 and the time integral of the receptor potential elicited by the same test flash after different delay times t_{DA}. Figure 6 shows a pronounced correlation for light responses at different states of dark adaptation. This relationship has already been reported by Maaz and Stieve (1980) for variation of light intensity and for a small variation in the state of adaptation. Sometimes we obtained two different slopes for small and large responses instead of the single slope shown in Fig. 6. The break between first and the second part of this relation corresponds to the first and the second phase observed in the course of dark adaptation (Fig. 5). Time integral and t_2 show the same behavior.

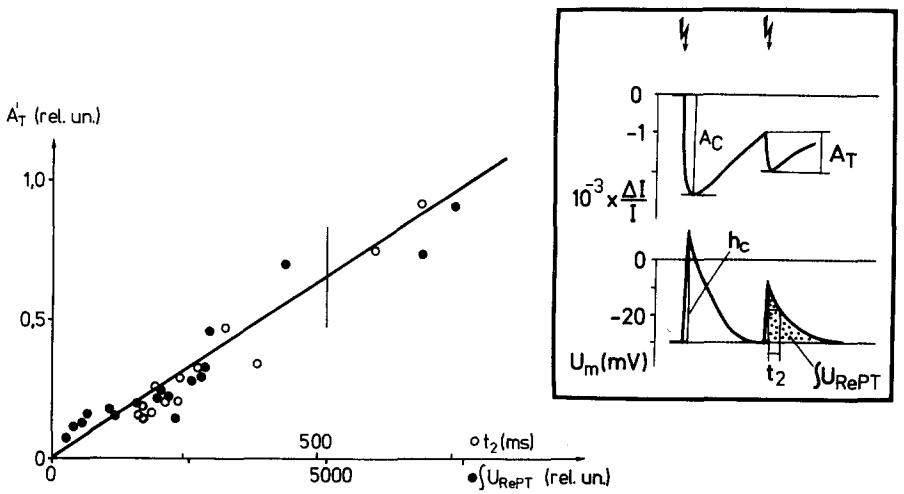


Fig. 6. The amplitude of the test flash-induced Arsenazo signal A'_T versus the half time of decay t_2 (open circles) and the time integral of the ReP (filled circles). The bar indicates the noise amplitude. Values are obtained from the experiment presented in Figs. 4 and 5. *Limulus* VNP

Voltage Clamp Experiments

It is not yet clear whether the measured light induced increase in the intracellular calcium ion concentration is the result of calcium influx, or whether the calcium is released from intracellular stores. Brown and Blinks (1974) supposed that in *Limulus* ventral nerve photoreceptor calcium comes partly from the outside into the cell. On the other hand Maaz and Stieve (1980) concluded that the major part of the light induced intracellular calcium increase originates ultimately from external sources, because it was significantly smaller in low extracellular Ca^{2+}. We carried out five voltage-clamp experiments with Arsenazo injected photoreceptor cells. The stimulus program is shown in Fig. 2. Some representative responses of a series are shown in Fig. 7.

The light induced receptor current ReC generally shows (as the ReP) the two components C_1 and C_2 of the light response, which can be observed clearly in Fig. 7, when the cell is clamped to negative potentials. Sometimes the components cannot be distinguished unambiguously. A clear appearance of the two components in the light induced current is rare when cells are tested under physiological circumstances without injected Arsenazo (Maaz et al. 1981). The more frequent obvious appearance of the two components may be due to the intracellularly injected Arsenazo III which may somewhat change the intracellular Ca^{2+}-concentration.

The Arsenazo response depends upon the membrane potential. When the membrane potential is clamped to negative potentials the Arsenazo response has a similar shape to that in unclamped cells (Fig. 7). However positive clamping (above +30 mV) causes a pronounced change of the time course of the Arsenazo response. The light induced Arsenazo signal becomes slower and even after switching off the clamp voltage the transmission returns extremly slowly to the prestimulus level (Fig. 7, lower right, U_m = +40 mV).

The amplitude of the light induced membrane current and the relative amplitude A'_{CL} of the Arsenazo response of a cell is plotted as function of the

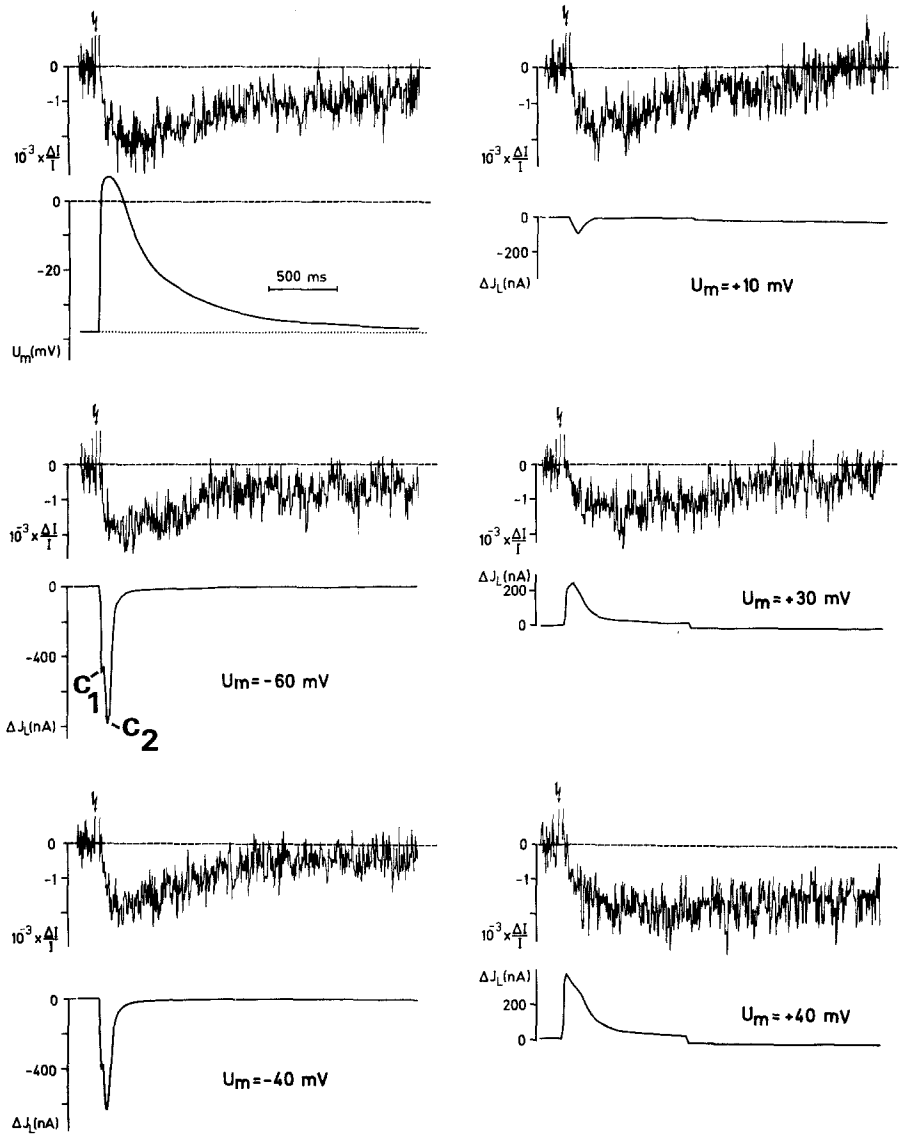


Fig. 7. Recordings in voltage clamp experiments. The upper left pair of curves indicates the first unclamped (free) response. U_m indicates the clamp potential. Each clamp cycle was followed by an unclamped (free) one (not shown). The cell was excited every 90 s with a 10 ms flash (light intensity 7 mW/cm^2). In each set the upper curve is the transmission (Fig. 2) signal, the lower curve is the membrane voltage – ReP or membrane current signal – ReC. The time scale is identical in all sets. C_1 and C_2 the two components of the ReC. *Limulus* VNP

membrane voltage in Fig. 8a and b. (The Arsenazo responses obtained in the clamped situation are normalized with respect to the unclamped responses preceding each clamping period.) The light induced current-voltage relation shows a linear relation in the potential range of U_m between -40 and $+40$ mV resulting in a slope conductance of ca. 14×10^{-6} S.

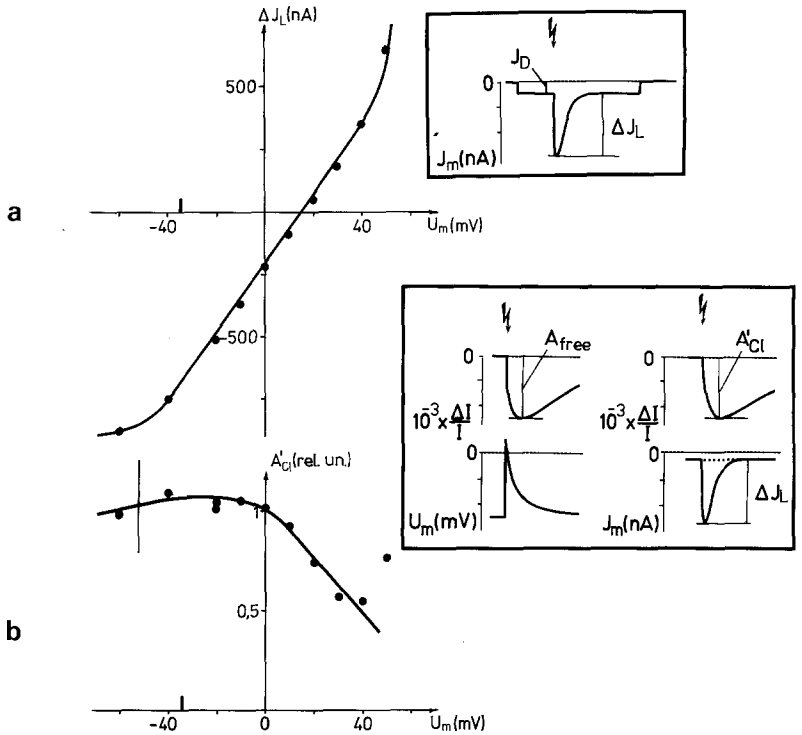


Fig. 8. The light induced membrane current ΔJ_L (curve a) and the normalized Arsenazo response A'_{CL} (curve b) as a function of membrane voltage (U_m). The bar at −38 mV indicates the prestimulus membrane potential PMP. The vertical bar indicates the noise amplitude. Same experiment as in Fig. 7. *Limulus* VNP

Outside of this interval the slope conductance deviates strongly from this value. Figure 8b shows that between −60 mV and zero the Arsenazo response depends only slightly upon membrane potential. However stronger depolarization of the cell membrane has a pronounced effect: the amplitude of the Arsenazo response is reduced by nearly one half, when the cell is clamped to +30 mV. Clamp potentials more positive than +40 mV cause changes of the amplitude and shape of the Arsenazo signal. The dependence of the Arsenazo signal upon the membrane voltage differed in other experiments in the positive voltage range (Ivens 1982; Ivens and Stieve 1981).

Plotting the relative height A'_{CL} of the Arsenazo signal as function of the light induced charge transported through the membrane under voltage clamp (time integral of ReC) gives a linear relation (Fig. 9). (This curve also shows a deviation from the linear relation at +40 mV.)

This indicates that the Arsenazo response (and this means the light induced increase in intracellular calcium ion concentration) is linearly proportional to the light induced charge transfer through the membrane of the visual cell.

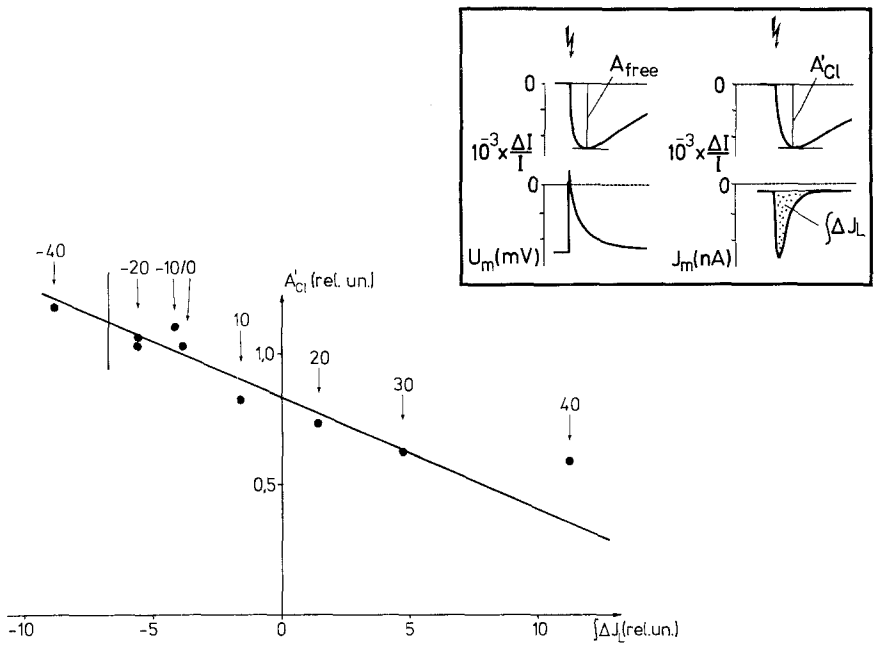


Fig. 9. Correlation of Arsenazo response and light induced charge transported across the cell membrane. Normalized Arsenazo response A'_{CL} obtained under voltage-clamp conditions as a function of the time integral of the light induced membrane current signal ΔJ_L. Numbers above the points indicate the corresponding clamp potential (U_m) for each record. The vertical bar indicates the normalized noise amplitude. Points are obtained from experiment presented in Figs. 7 and 8. Light intensity was const. = 7 mW/cm^2. *Limulus* VNP

Discussion

Brown et al. (1977) have shown that under appropriate conditions the absorption change of the Arsenazo III in *Limulus* photoreceptors is caused exclusively by the change in calcium ion concentration. So the Arsenazo response obtained by us is a measure of the transient increase in calcium ion concentration in the cell. The effect of other ions (Mg^{2+}, H^+) and of different factors, which can influence the absorption of the dye (carefully analyzed by Brown et al. 1977; Brown and Pinto 1979) are negligible under our experimental conditions.

It may be that the injected Arsenazo III has an effect on the properties of the dark adaptation of the cell due to its high binding constant to Ca^{2+} (Ohnishi 1979). However, it seems improbable that injection of Arsenazo causes a functional change in the adaptation behavior of the cell, though it might accelerate the adaptation process.

We observed that there are active spots in the cell, where the light induced calcium concentration change is much higher than in other regions. Two possibilities can explain this phenomenon: a) the Arsenazo could be inhomogeneously distributed in the cell and/or b) there are calcium active regions in the

cell, probably close to the photosensory membrane where the primary light induced increase in calcium concentrations occurs and from which other regions are affected. The second supposition seems more probable to us in accordance with conclusion of Meech and MackBrown (1976) and Maaz and Stieve (1980) who supposed that the calcium active areas may be located in the neighbourhood of the microvillar membrane.

Our results show that the maximum increase in the calcium ion concentration after a light flash occured much faster when smaller amounts of Arsenazo were injected (compare Maaz and Stieve 1980). The intracellular calcium ion concentration influences the state of adaptation of the cells; because the more Arsenazo which is injected the more the light response is retarded, probably due to the buffer effect of Arsenazo. In intact cells the calcium increase is probably even faster than observed here. We assume that the calcium permeable channels are opened at approximately the same time after a light flash, as (or are identical with) the light activated sodium preferring channels.

Our voltage clamp experiments indicate that changes in the membrane potential especially those to positive values have a strong effect on the flash-induced increase in calcium concentration. The transient increase in intracellular Ca^{2+}-concentration is a linear function of the total transported charge (see Fig. 9), which suggests that calcium influx may be a constant fraction of the total ion influx. Ivens and Stieve (1981) and Ivens (1982) found that the Arsenazo response at normal or low (250 μmol/l) external calcium ion concentration depended upon the membrane voltage under voltage clamp. By extrapolation they derived reversal potentials for the Arsenazo response which depended upon the external calcium concentration and were equal to or more positive than the Nernst potential for calcium.

These findings suggest besides those mentioned by Stieve and Bruns (1980), that at least a major part of the light induced increase in $[Ca^{2+}]_i$ is due to a Ca influx through the cell membrane during the electric light response.

Our results obtained investigating dark adaptation (Fig. 5) are in good agreement with those concerning the dark adaptation of the receptor potential components C_1 and C_2 (see Maaz et al. 1981). These two components of the receptor potential can be separated during dark adaptation because they recover differently fast. Our present observations of the recovery of the intracellular calcium signal show a similar behavior.

The calcium influx could be involved in the first (C_1) and/or in the second (C_2) component of the membrane current (Fig. 7). Neither C_1 nor C_2 can be a pure calcium current: Their reversal potentials are between +16 mV and +26 mV. This is much below the equilibrium potential for calcium which is around +140 mV. The reversal potentials of the two components showed a difference of 6–8 mV, which was not statistically significant (Maaz et al. 1981). It could be that the two components C_1 and C_2 of the receptor potential are based on two different populations of light activated ion channels of similar type which are located in different neighbourhood, for instance the internal and the outer rhabdom which was described by Calman and Chamberlain (1982).

The light induced Arsenazo response recovers much stronger in the second phase of the dark adaptation than in the first phase, in contrast to the amplitude

of the ReP which grows to almost 90% during the first phase of dark adaptation (Fig. 5).

The two phases of dark adaptation result from the different rates of recovery of the two components C_1 and C_2 of the ReP (Maaz et al. 1981; Claßen-Linke and Stieve 1981; Claßen-Linke 1981). The different ratios of recovery of receptor potential and Arsenazo signal during the two phases of dark adaptation indicate that the Ca-influx accompanying C_2 is relatively larger than that accompanying C_1. Since the peak voltage h_T of the light response was under our experimental conditions in the second phase relatively close to the reversal potential of the light response, the relative rise in amplitude of the two phases of dark adaptation do not provide an estimate of the ratio of the underlying increase in membrane conductance.

During the recovery to the dark adapted state the decrease of the prestimulus level A'_P of intracellular calcium shows an exponential decay during the first 10 s (Fig. 5). According to Ivens (1982) active Ca^{2+} transport is at least in part responsible for this decrease. During the first phase of dark adaptation there is a clear correlation between the recovery of the height of the receptor potential and the decline of the intracellular calcium concentration (Fig. 5). I.e., here the calcium may directly regulate the sensitivity of the photoreceptor. During the second phase of the recovery of sensitivity in dark adaptation no change in the prestimulus level A_P of $[Ca^{2+}]_i$ could be detected. This is a converse situation to that described by Brown and Blinks (1974) where during prolonged illumination a decrease in $[Ca^{2+}]_i$ was not accompanied by a change in membrane depolarization. It is possible that the second phase of dark adaptation could be regulated by a slow release of calcium from a regulatory binding site so that the time course of relaxation would be determined by a slower process than the decline in $[Ca^{2+}]_i$. Alternatively it may be that $[Ca^{2+}]_i$ is not the regulator of sensitivity during the second phase of dark adaptation.

Claßen-Linke and Stieve (1981), measured the sensitivity of *Limulus* photoreceptors in the course of dark adaptation by determining the stimulus intensities which evoked criterion response amplitudes. They showed that the first phase of dark adaptation depends strongly on the extracellular calcium concentration whereas the following second phase of dark adaptation is nearly Ca-independent. Perhaps the calcium-dependent regulation of sensitivity is a threshold phenomenon as suggested by Claßen-Linke (1981) and Stieve (1982). If $[Ca^{2+}]_i$ is higher than a certain threshold level it regulates the sensitivity of the photoreceptor cell (first phase of dark adaptation); below this level (second phase), another, at present unknown, process determines its sensitivity.

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