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

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Limulus Ventral Nerve Photoreceptor, Voltage Clamp, Membrane Voltage Dependence of the Light-Induced Arsenazo III Signal, Ca^{2+}-Concentration Change

The light induced transmission change (Arsenazo signal) of an Arsenazo III injected ventral photoreceptor cell of *Limulus polyphemus* was studied under voltage clamp. The transmission change which represents a change of free intracellular calcium ion concentration, $[Ca^{2+}]_i$, was investigated for its dependence upon membrane voltage.

The peak amplitude of the Arsenazo signal decreases in a linear fashion with the clamp voltage in the examined voltage range (from − 80 to + 40 mV).

In low Ca^{2+} saline ($[Ca^{2+}]_e = 250$ µM) this decrease in the amplitude of the Arsenazo signal was more pronounced, while in saline with increased Ca^{2+} ($[Ca^{2+}]_e = 40$, 50 and 100 mM), there is almost no change of the Arsenazo signal with varied membrane voltage.

The recovery of the Arsenazo signal (*i.e.* recovery of the transmission back to the value before the light flash) is faster during hyperpolarization, this recovery being slowed down when the cell is depolarized.

From these experiments it is concluded that a substantial part of the Arsenazo signal is due to a light induced influx of Ca^{2+} from the extracellular space across the cell membrane into the cytoplasma. Conceivably the Ca^{2+} could pass through light activated Na^+ channels. Subsequently the increased intracellular Ca^{2+} is lowered to the preillumination level, by a membrane voltage dependent mechanism possibly an $Na^+ - Ca^{2+}$ exchange.

The data do not exclude the possibility that a part of the Ca^{2+} responsible for the Arsenazo signal is released from intracellular stores.

Abbreviations

ReP	receptor potential (light induced membrane voltage signal);
ReC	receptor current (light induced membrane current signal under voltage clamp);
PMP	prestimulus membrane potential;
V_C	clamped membrane voltage;
A_{free}	peak amplitude of Arsenazo signal induced in the unclamped (free cell) and
A_C	under voltage clamp conditions;
$A'_C = \frac{A_C}{A_{free}}$	normalized Arsenazo signal;
A_{CE}	height of the clamped Arsenazo signal 6 s after the light flash;
A_{FE}	height of the unclamped Arsenazo signal 6 s after the light flash;
$A'_{CE} = \frac{A_{CE}}{A_{FE}}$	normalized height of Arsenazo signal 6 s after the light flash;
PS	physiological saline;
$[Ca^{2+}]_e$	extracellular calcium ion concentration;
$[Ca^{2+}]_i$	intracellular calcium ion concentration.

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Introduction

The influence of calcium ions on the light response of the ventral eye of *Limulus* polyphemus has been studied for a number of years by various authors [1–6]. Variation in the extracellular calcium concentration of the photoreceptor superfusion medium, revealed the first evidence for the sensitivity of the light response to this ion species. Measurements with intracellularly injected Aequorin showed directly following illumination an increase of intracellular free Ca^{2+} [1]. Similar findings were obtained with the metallochromic calcium indicator Arsenazo III [2, 7, 8]. The experiments on the ventral photoreceptor injected with Arsenazo III showed that 20 to 50 ms after a response saturating light flash the intracellular free Ca^{2+} increases and returns during the dark adaptation period to the preillumination level. The contribution of Ca^{2+} to the light induced photocurrent is found to be small under voltage clamp conditions [9, 10].

This paper presents a voltage clamp study of the light induced increase in intracellular Ca^{2+} using

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Arsenazo III as an intracellular monitor. The measurements show that the Arsenazo signal depends on the membrane voltage and that a Ca^{2+} reversal potential, if present, must be much larger than + 60 mV, the reversal potential for sodium. A hypothetical reversal potential for the Arsenazo signal, estimated by linear extrapolation, depended upon the external Ca^{2+} concentration. In 10 mM $[Ca^{2+}]_e$ it was always larger than + 250 mV.

Materials and Methods

The ventral nerve of *Limulus* polyphemus was isolated and treated with pronase [11]. For the experiments the nerve was fixed with steel needles onto a piece of sylgard in a glass chamber. During the experiments the chamber was continuously superfused with saline of constant temperature (17 °C). The following salines (pH 7.5) were used (Table I).

The osmolarity of R_1, R_2 and R_3 used in the experiments with high extracellular Ca^{2+}-concentration was adjusted by sucrose to the osmolarity of the Ca^{2+} saline. The ventral nerve was first incubated in the appropriate PS for 30–60 min before switching to the high Ca^{2+} media.

During the experiments the Arsenazo signal (A) and receptor current (ReC) or receptor potential (ReP) were recorded simultaneously. The setup was essentially the same as recently described [8] except that the transmission changes of the receptor cells were detected by a sample and hold technique as described by Spalink [12].

The receptor cell was stimulated every other minute by a saturating white light pulse of 10 ms duration (Hg high pressure lamp HBO 100; Osram). The light intensity in the vessel was 1.1 mW cm^{-2}. The continuous measuring light (ML: $\lambda_{max} = 645$ nm, Schott interference filter; half width 9 nm) was focussed onto the receptor cell, so that a maximal cell area was illuminated and recorded by a photomulitplier. The diameter of the light beam could be varied between 30 and 200 µm. In order to exclude the influence of hot spots of Ca^{2+} [8] the whole cell body was illuminated by the measuring light beam. The light intensity of the measuring light was between 1 and 10 µW cm^{-2}. The red ML reduced the prestimulus membrane potential (PMP) maximally by 2 mV.

Every alternate flash stimulus, the membrane voltage of the cell was clamped to various voltages for 9 s. The clamp started 3 s prior to the flash and the light induced receptor current was measured simultaneously with the Arsenazo signal (Fig. 1). The membrane voltage of the cell was initially clamped at −80 mV subsequently the clamp voltage was increased in steps of + 10 mV. One minute after each clamped light response the receptor potential together with its corresponding Arsenazo signal was recorded. The Arsenazo signal (clamped and unclamped), ReC and ReP were averaged with the corresponding signal induced two minutes later under the same conditions.

For calculation of the normalized Arsenazo signal A'_C the peak amplitude of the averaged clamped Arsenazo signal A_C is divided by that of the averaged Arsenazo signal of the unclamped cell.

$$A'_C = \frac{A_C}{A_{free}}.$$

The division of the peak amplitude of the clamped cell by the shortly afterwards induced signal of the free cell was done to exclude any slow changes of the Arsenazo signal due to diffusion of the Arsenazo III or changes in the receptor cell because of the long duration of the experiment.

Table I. Salines of different calcium-concentrations used in the experiments.

NaCl [mmol/l]	KCl [mmol/l]	$CaCl_2$ [mmol/l]	$MgCl_2$ [mmol/l]	$MgSO_4$ [mmol/l]	NaOH [mmol/l]	Hepes [mmol/l]	Sucrose [mmol/l]	
480.8	10	10	25	30	5	10	–	PS
495.4	10	0.25	25	30	5	10	–	250 µM Ca^{2+}
435	10	10	25	30	5	10	270	R_1
435	10	100	25	30	5	10	–	100 mM Ca^{2+}
480.8	10	10	25	30	5	10	90	R_2
480.8	10	40	25	30	5	10	–	40 mM Ca^{2+}
480.8	10	10	25	30	5	10	–	R_3
480.8	10	50	25	30	5	10	–	50 mM Ca^{2+}

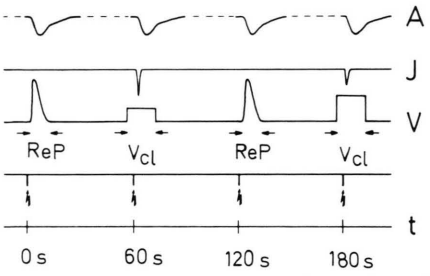

Fig. 1. Scheme of the experimental regime of clamp phases correlated with light stimuli. The receptor cell was stimulated by a 10 ms light flash every minute. Every second minute the cell was clamped for 9 s starting 3 s before the light flash. The same clamp voltage was repeated 180 s later and these two signals were averaged. ReP: receptor potential; A: Arsenazo signal; J: membrane current = ReC; V_{CL}: clamped membrane voltage; t: time.

The same division was performed for the averaged height of the Arsenazo signal A'_{CE} 6 s after the light flash

$$A'_{CE} = \frac{A_{CE}}{A_{FE}}.$$

For the microelectrodes theta glass tubes pulled to a tip resistance of 10 to 20 MΩ, were used. The current electrode was filled with 20 mM Arsenazo III in 0.5 M KCl, pH 7.1 (Arsenazo III was purified according to Bauer, [13]). The sensor microelectrode contained 0.5 M KCl. Membrane current was recorded via an $Ag^+/AgCl$ electrode connected to the vessel by an agar salt bridge. Arsenazo III was pressure-injected into the photoreceptor cell 30 min before the measurements began, in order that the dye should diffuse freely throughout the cell.

Upon changing the extracellular saline, 30 min were allowed for stabilization of the light response. Maintaining low levels of injected Arsenazo III ensured that no changes in the PMP and ReP resulted. During the experiments which usually lasted 4 h the amplitude of the Arsenazo signal diminished slowly (on average by less than 10% of the starting value) possibly by dye diffusion into the axon.

Results

The transmission of an Arsenazo III injected photoreceptor cell of *Limulus* is transiently reduced following a light flash [2, 7, 8]. Comparable to the findings of Brown and Blinks with injected Aequorin ([1]) the light induced transmission change is also present in a voltage clamped photoreceptor containing Arsenazo III [8]. The Arsenazo signal consists of an early decrease in transmission with a maximum 100 to 800 ms after the light flash (Fig. 2). This decrease is followed by a slower increase in transmission back to the dark level. The whole signal lasting between 10 and 60 s after an amplitude saturating light flash of the receptor potential. Brown *et al.* [2] showed that the transmission decrease is the result of an increase in intracellular free Ca^{2+}. The following transmission return is due to a lowering of Ca^{2+} back to the dark adapted level.

The voltage clamp experiments described here compare the differences between the Arsenazo signal of the unclamped and the clamped receptor cell (Fig. 2).

The peak amplitude of the Arsenazo signal (A_C) is reduced at depolarizing clamp voltages and increased at hyperpolarizing voltages. This is shown by a plot of the normalized Arsenazo signal A'_C versus the clamp voltage (Fig. 3a). The correspond-

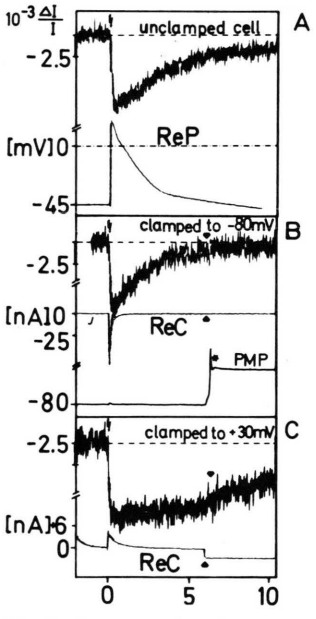

Fig. 2. Arsenazo signals (clamped and free), receptor potential (ReP) and receptor currents (ReC) of the ventral photoreceptor. A: Arsenazo signal and ReP of the unclamped cell (two signals averaged). B: Arsenazo signal, ReC and membrane voltage (V_C) of the cell clamped to − 80 mV (two signals averaged). (After the voltage clamp is switched off (arrows) the membrane potential shows an overshoot (asterisk).) C: Arsenazo signal and ReC of a cell clamped to + 30 mV (two signals averaged). Exp. 7.

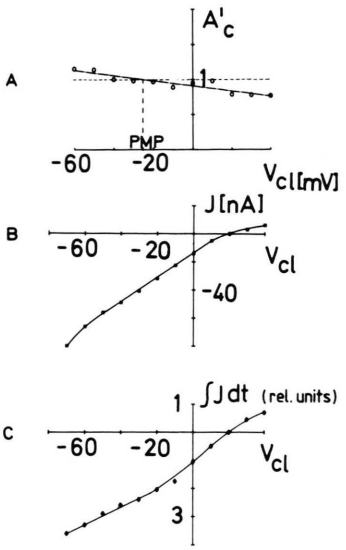

Fig. 3. A: The normalized amplitude $A'_C = A_C/A_{free}$ of the Arsenazo signal (relative units) is plotted *versus* the clamp voltage (V_C). The reversal potential of the Arsenazo signal (point of intersection with the abscissa) is calculated by linear extrapolation to be + 250 mV. The dashed line parallel to the abscissa indicates the height of the unclamped signal (*i.e.* 100%). PMP is the prestimulus membrane potential. B: maximal amplitude of the light induced receptor current (ReC) *versus* V_C. C: time integral of the ReC (relative units) *versus* V_C. Exp. 7.

ing height of the light induced receptor current and the time integral of the current is seen in Fig. 3b and c. The values in Fig. 3a were fitted by linear regression (plotted line) and the intersection point with the abscissa calculated to be + 250 mV. In 12 experiments the same fit was performed with the calculated intersection points ranging between + 250 and + 961 mV ($\bar{x}$ = 640 mV). No reversal potential for A_C could be measured up to + 60 mV. The decrease of A_C was present in all experiments even when the clamp was started at positive voltages and proceeded to the negative.

Nagy and Stieve [8] reported, based on 3 experiments in which the entire membrane voltage range from − 40 to + 20 mV was tested, a somewhat different dependence of the Arsenazo signal amplitude from the clamped membrane voltage. We think that the data reported here are more typical: Only one out of the 19 experiments on which this publication is based, showed a similar behaviour as that described in [8]. The other 18 experiments gave results similar to Fig. 3.

In 5 experiments the extracellular Ca^{2+} was reduced from 10 mM (PS) to 250 μM. This procedure reduced the amplitude of the unclamped Arsenazo signal (A_{free}) to 65% (± 13%; n = 4). The Arsenazo signal is prolonged similar to ReC and ReP (Fig. 4). In one experiment no Arsenazo signal was detectable after the saline was changed to low Ca^{2+} (see also [7]).

The normalized Arsenazo signal in low $[Ca^{2+}]_e$ differs strongly from the signal of the same cell in PS (see Fig. 5a and b). A'_C in low Ca^{2+} is reduced drastically and the slope of the regression line is steeper. The calculated intersection points were at lower voltages than in PS ($\bar{x}$ = 110 mV ± 21 mV; n = 4).

To see the influence of increased extracellular Ca^{2+}-concentration, the concentration of Ca^{2+} was changed in 3 experiments to 100 mM. In two of the experiments the Arsenazo signal disappeared within 30 min. The other experiment showed no change of A'_C with increased clamp voltage while the amplitude of the signal (A_{free}) was reduced to 91% of the amplitude in PS. In two out of 3 experiments the Arsenazo signal was reduced to zero by a Ca^{2+} concentration of 50 mM. The amplitude of the third experiment decreased to 88%. In salines with 40 mM Ca^{2+} 3 experiments were done. A_{free} diminished to 65% (± 4; n = 3) of the value in PS.

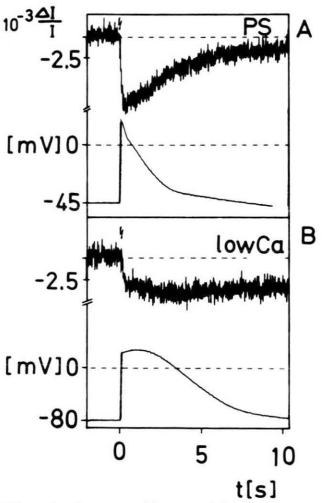

Fig. 4. In a saline with low external Ca^{2+} concentration Arsenazo signal, ReC and ReP are prolonged. A: Arsenazo signal of the unclamped cell and ReP of a cell in PS (two signals averaged). B: Arsenazo signal and ReP of an unclamped cell superfused for 20 min with saline containing 250 μM Ca^{2+} (two signals averaged). Exp. 9.

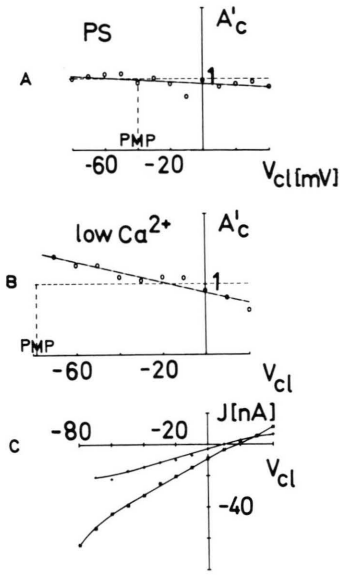

Fig. 5. A: The normalized amplitude A'_C of the Arsenazo signal (relative units) of the photoreceptor in physiological saline with 10 mM Ca^{2+} (PS) is plotted *versus* clamp voltage. The decrease of A'_C with increased membrane voltage is significant and was seen in every experiment with salines containing normal or reduced Ca^{2+}. The extrapolated reversal potential is + 770 mV. B: A'_C (relative units) of the same cell in a saline with 250 μM Ca^{2+}. The extrapolated reversal potential is calculated to be + 122 mV. C: ReC of the cell in PS (*, lower line) and in low Ca^{2+} (+, upper line) *versus* clamped membrane voltage. Exp. 9.

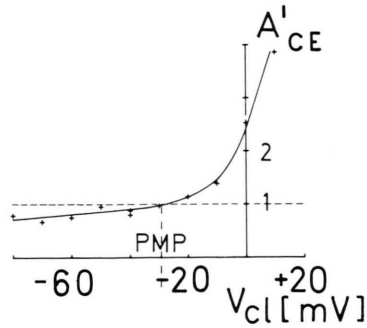

Fig. 6. The return of the Arsenazo signal back to the dark level depends strongly on the membrane voltage. The normalized height of the Arsenazo signal 6 s after the light flash (A'_{CE} relative units) plotted *versus* clamped membrane voltage. Photoreceptor in PS. Exp. 10.

The normalized Arsenazo signal (A'_C) in high Ca^{2+} showed a strong variation. Only in one experiment (100 mM Ca^{2+}) could the calculation for A'_C be done with any accuracy. A_C did not decrease with increased membrane voltage and the calculated regression line was nearly parallel to the abscissa. It seems that the dependence of the Arsenazo signal on membrane voltage is reduced when the external Ca^{2+}-concentration is raised above the physiological level.

Besides the maximum of the Arsenazo signal the transmission increase back to the dark level depends on the membrane voltage of the photoreceptor (Fig. 2b, c). The height of the clamped Arsenazo signal in PS, 6 s after the light flash (A_{CE}) was measured and divided by the height of the unclamped signal (A_{FE}) 6 s after light to normalize the values:

$$A'_{CE} = \frac{A_{CE}}{A_{FE}}.$$

In Fig. 6 A'_{CE} is plotted versus the membrane voltage. During hyperpolarizing clamp voltages the increase of the signal back to the dark adapted level is faster (Fig. 2b) and during depolarization it is slower (Fig. 2c) than in the unclamped Arsenazo signal (Fig. 2a).

At large depolarizations the Arsenazo signal of same cells did not increase after the first transient decrease but decreased slowly during the depolarization step (data not shown).

Discussion

For the ventral photoreceptors of *Limulus* Ca^{2+} was found to control light and dark adaptation processes [3, 14–16]. With the intracellular chemoluminescent protein Aequorin [1] and the Ca^{2+} binding dye Arsenazo III [2] the relative changes in intracellular Ca^{2+}-concentration could be demonstrated.

The absorption spectrum of Arsenazo changes in the presence of Ca^{2+}. It is also influenced by pH and other divalent cations (*e.g.* Mg^{2+}). The affinity of Arsenazo III for Mg^{2+} is nearly two orders of magnitude smaller than for Ca^{2+} [17]. For the ventral photoreceptor of *Limulus*, Brown *et al.* [2] have shown that the absorption spectrum of intracellularly injected Arsenazo III represents the binding of only intracellular free Ca^{2+} to the probe. The injection of Mg^{2+} together with Arsenazo III exhibited a different absorption spectrum to that of a light adapted receptor cell containing Arsenazo III. Intracellular pH changes of the receptor cell following saturating

light flashes were shown to be smaller than 0.1 pH units [2].

The voltage clamp experiments with Arsenazo III from Nagy and Stieve [8] and our data shown that a light induced increase of free Ca^{2+} in the ventral photoreceptor of *Limulus* is not due to voltage triggered membrane channels. They also show that the Arsenazo signal of the photoreceptors is not due to voltage triggered Ca^{2+} release out of intracellular stores, as seen in skeletal muscle [18].

To study the influence of membrane voltage on the transient increase of $[Ca^{2+}]_i$ due to light, we did several experiments with varied extracellular Ca^{2+} concentrations. In the region in which the membrane voltage was varied, there appears to be a linear decrease in the amplitude of the normalized Arsenazo signal (A'_C) with physiological saline and lowered extracellular calcium concentration (Figs. 3a, 5a, b). This is especially notable in cells superfused with low Ca^{2+} containing saline.

From plots of the clamp voltage versus relative amplitude of the Arsenazo signal (A'_C) a regression line was calculated and point of intersection on the abscissa obtained by extrapolation. The calculated point is only a measure for the different steepness of the regression lines. This point should correspond to the Ca^{2+} reversal potential for the light induced Ca^{2+} influx, calculated by the Nernst equation if all the Ca^{2+} enters from the extracellular space driven by the electrochemical Ca^{2+} gradient across the cell membrane.

It was found that the extrapolated reversal potential for the Arsenazo signal in PS and in media containing increased Ca^{2+}-concentrations is in each case far more positive than the values predicted from the Nernst equation (*i.e.* + 158 mV) for the estimated Ca^{2+} gradient. From the data it can be said, that the reversal potential for the Arsenazo signal is at least more positive than ca. + 15 mV, the measured reversal potential for the light induced receptor current [19] and also more positive than + 60 mV, the Nernst potential for sodium. This excludes the possibility that a light induced sodium influx induces the observed Ca^{2+} increase in the receptor cell [3].

In saline of low Ca^{2+}-concentration a Ca^{2+} reversal potential of + 98.5 mV can be calculated from the Nernst equation ($[Ca^{2+}]_i = 10^{-7}$, $[Ca^{2+}]_e = 2.5 \times 10^{-4}$ M). This is close to the reversal potential extrapolated from the measured decrease of the Arsenazo signal (A'_C) (see Fig. 5).

It is conceivable that in saline containing normal or high Ca^{2+} concentration the behavior of the cell membrane is not adequately described by the Nernst equation. There may be processes contributing to the light induced Arsenazo signal (*e.g.* intracellular stores *c.f.* [20, 21]) which are independent of the membrane potential and which are absent or reduced in low extracellular Ca^{2+}. This processes may simply shift the A'_C curves by a constant, voltage-independent value on the ordinate which is absent in low $[Ca^{2+}]_e$. One possibility is, that intracellular stores normally contribute to the Arsenazo signal. If the intracellular stores are empty at low extracellular Ca^{2+} due to lowered intracellular Ca^{2+}, their contribution to the Arsenazo signal is negligible.

In experiments with increased extracellular Ca^{2+} (40, 50 and 100 mM) the Arsenazo signal disappears completely in 4 cells and in the other 5 experiments the Arsenazo signal was reduced and showed strong variations in amplitude showed only minor changes. As evidenced by a slow transmission decrease of the cell in the dark after increased $[Ca^{2+}]_e$ it was apparent in some of these experiments that increased extracellular Ca^{2+} increases the level of intracellular Ca^{2+}. It may be possible that the light induced increase of free Ca^{2+} is reduced because of the increased level of intracellular Ca^{2+} and therefore in some experiment not detectable.

This finding is in agreement with the measurements of free intracellular Ca^{2+} in *Limulus* ventral photoreceptor using Ca^{2+} sensitive microelectrodes [22, see also 23]. Owen found that the intracellular free Ca^{2+} in dark adapted photoreceptors of *Limulus* was 3×10^{-7} M. Light adaptation increases the concentration to 7×10^{-7} M. In high extracellular Ca^{2+} concentration he found no increase due to light, while he saw an increase from 3 to 7×10^{-7} M in normal saline.

The voltage clamp experiments described here indicate that at least a substantial fraction of the Ca^{2+} increase due to light is driven by the membrane voltage and concentration gradient across the receptor cell membrane. Ca^{2+} must enter the cell through the light activated sodium channels [5, 24] or through special light sensitive Ca^{2+} channels. Voltage dependent Ca^{2+} channels recently found in the ventral nerve photoreceptor [9, 10] can not contribute to this Arsenazo signal because the volt-

age clamp is started already 3 s before the light pulse.

Without light activation, only due to voltage steps a transmission change was not observed in our measurements. This indicates that under the experimental conditions applied here the voltage dependent Ca^{2+} increase [9, 10] does not influence significantly the Arsenazo signal of the free cell. Intracellular Ca^{2+} stores which may contribute to the Arsenazo signal [1, 21] can not be excluded but should not be influenced by the membrane voltage.

The increase of transmission back to the dark level depends strongly on the membrane voltage (Figs. 2, 6). This is explicable in terms of a membrane voltage sensitive mechanism for Ca^{2+} outward transport through the receptor cell membrane. It should not apply for a Ca^{2+} transport into intracellular compartments. In other tissues a membrane voltage dependent Na^+/Ca^{2+} exchange mechanisms has been suggested in the cell membrane [24–28]. By hyperpolarization of the cell membrane the Ca^{2+} transport out of the receptor cell is stimulated whilst depolarization slows it down (Fig. 2b and c). In some of the experiments at positive clamp voltages (*i.e.* $> +40$ mV) the amplitude of the Arsenazo response of the photoreceptor decreases slowly during the whole clamped phase after the normal transient first rise. (Data not shown.)

These findings indicate that there must be at least one other ion involved in this process since Ca^{2+} transport out of the cell should be slowed down by hyperpolarization. It seems possible that there is an antiport by which Ca^{2+} is transported out of the photoreceptor and another cation (*e.g.* Na^+) enters the cells in a stoichiometric ratio. The possibility of a co-transport system of Ca^{2+} with an anion *e.g.* Cl^- seems at present unlikely.

In voltage clamp experiments with Arsenazo III injected receptor cells at least two indications were found that a large part of the intracellular Ca^{2+} increase due to light enters the cell through the cell membrane: voltage dependence of the amplitude of the Arsenazo signal and voltage dependence of the increase of the Arsenazo signal back to the dark level. It can be assumed that a substantial part of the light induced increase of intracellular Ca^{2+} enters through the outer membrane but at present we can not estimate how large the fraction is.

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