The Light-Stimulated Cytosolic Calcium Transient in *Limulus* Ventral Nerve Photoreceptors: Two Components in the Rising Phase

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1. Light-evoked electrical responses were measured in *Limulus* ventral nerve photoreceptors simultaneously with changes in the cytosolic free calcium concentration, by means of arsenazo III.

2. It has been shown here for the first time that the rise of the arsenazo signal consists of two phases. Only the slow phase in the rise of the signal depends on the membrane voltage. The reversal potential of the amplitude of this slow rising phase was about +196 mV. After removal of external calcium the reversal potential was about +20 mV.

3. When Na^+ in the superfusate was replaced by Li^+, the amplitude of the fast rising phase was reduced on the average to 50%, while the slow rising phase was not affected.

4. We conclude that the fast rising phase is caused by release of calcium from internal stores while the slow increase in $[Ca^{2+}]_i$ is due to influx across the plasma membrane, possibly through light-activated ion channels.

Introduction

In *Limulus* ventral photoreceptors light causes an electrical response accompanied by an increase in the intracellular free calcium concentration $[Ca^{2+}]_i$. The rise in $[Ca^{2+}]_i$ after light stimulation is known to mediate light adaptation (Lisman and Brown, 1972; Lisman and Brown, 1975; Stieve, Bruns and Gaube, 1984), and probably it is a messenger of light excitation, too (Payne, Corson and Fein, 1986). It is well established that inositol 1,4,5-trisphosphate (IP_3) releases calcium from intracellular stores (for review see Fein and Payne, 1989; Nagy, 1991). The internal stores are refilled by ATP-dependent uptake of calcium from the cytosol (Walz and Fein, 1983). For photoreceptors of barnacle (Werner, Suss-Toby, Rom and Minke, 1992), honey bee (Minke and Tsacopoulos, 1986; Ziegler and Walz, 1989) and fly (Hardie, 1991; Ranganathan, Harris, Stevens and Zuker, 1991), an additional source of calcium has been reported. In these preparations calcium permeates light-activated ion channels in the plasma membrane. For *Limulus* ventral photoreceptors the existence of light-activated calcium-permeable channels is controversially discussed. So far there has been no direct evidence for this hypothesis (Brown and Mote, 1974; Deckert and Stieve, 1991). Besides a possible influx of calcium through channels, a Na-Ca exchanger is a pathway for calcium transport across the plasma membrane. The existence of a Na-Ca exchanger in the plasma membrane of *Limulus* ventral photoreceptors has been shown by O'Day and Gray-Keller (1989). This exchanger is able both to export and import calcium across the plasma membrane (Deckert and Stieve, 1991).

The present study gives evidence for calcium influx through light-activated ion channels in the plasma membrane of the ventral photoreceptor. Our data show that the light-stimulated transient increase in $[Ca^{2+}]_i$ consists of two phases which can be described by the sum of two exponentials. The arsenazo signal starts with a fast phase and reaches its maximum with a slow further increase. The fast phase is not detectably influenced by changes in the membrane voltage. It is reduced after replacement of sodium by lithium in the superfusate. When calcium is removed from the superfusate, the fast rising phase of the arsenazo signal is drastically decreased after repetitive illumination. We suggest that this phase is caused by the release of calcium from internal stores, the submicrovillar cisternae (see also Deckert and Stieve, 1991). The slow phase on the other hand depends on the membrane voltage and is not af-

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fected after replacement of sodium by lithium in the superfusate. After reduction of external calcium the slow phase persists even after 10–20 cycles of test flashes. The amplitude of the slow phase is reduced when the cells are voltage clamped to depolarizing membrane voltages. The reversal potential is shifted to more negative values compared to results measured in physiological saline. This indicates calcium influx across the plasma membrane probably through light-activated channels.

Parts of this study have been published as abstract (Stommel, Rüsing, Yuan and Stieve, 1994; Stommel and Stieve, 1995).

Methods

The experimental technique was similar to that described by Deckert and Stieve (1991). Ventral nerves were dissected from male *Limulus polyphemus* (carapax width: 15–25 cm). The nerve was desheathed and fastened to a silicone rubber block and kept in physiological saline or in organ culture medium (Kass and Renninger, 1988) for 3–48 h. It was treated with collagenase (2 mg/ml physiological saline) for 10 min and placed in a glass chamber (1.5 ml volume) which was perfused with 1 ml physiological saline per minute at constant temperature (15 °C). The glass micropipette used for intracellular recording and arsenazo injection was filled with 0.5 M KCl, its tip containing 20 mmol/l arsenazo III dissolved in 0.5 M KCl. The electrode resistance was between 3 and 18 megohm. Most of our electrical recordings were done with the two electrode voltage clamp (2 VF-180, CA-100, 2 HS-170 Biologic, France). In these experiments arsenazo was filled into the driver electrode. In some experiments we additionally measured receptor currents with the one electrode voltage clamp (Deckert and Stieve, 1991). When results did not differ significantly, they were pooled. In lithium saline the receptor potential was measured, because lithium influences the dark potential of the cell. This would have led to large dark currents under constant voltage clamp conditions which might have damaged the cell. The receptor current (ReC) was measured by clamping a cell to the desired membrane potential 0.5 s before the onset of the light stimulus. This procedure was adopted to prevent a voltage-driven desensitization of the cell due to calcium influx through the Na-Ca exchanger while the cell was depolarized (Deckert and Stieve, 1991). Arsenazo III (Sigma) was injected into the cell with a pressure of 1–4 bar. A photometric beam (645 nm, half width 9 nm; intensity after passing the unstimulated cell I_m = 0.1–1 mW/cm^2; diameter approximately 125 μm) was focused onto the photoreceptor. This caused light adaptation, reducing the sensitivity of the cell 5–40 fold. To reduce this desensitizing effect, the photometric beam was controlled with an electromagnetic shutter which allowed the photometric beam to pass only within the recording period. The arsenazo signal reached a stable baseline about 200 ms after the opening of the shutter. A test flash was applied 300 ms after the onset of the photometric beam, i.e. when the baseline was already constant. Only experiments with lithium saline were carried out with steady state photometric beam, because the receptor potential caused by the onset of the photometric beam might have overlapped the response to the test flash. Intracellular changes of the cytosolic free calcium concentration lead to a transient transmission change of the cell, termed arsenazo signal, which was monitored by a photomultiplier, amplified, and low-pass filtered (100 Hz). The electrical response and the arsenazo signal were digitized at 1 kHz and stored by computer (for details see Deckert and Stieve, 1991). Cells were stimulated with test flashes of 1–2 ms half-width (515 nm maximum, wide band filter). Light intensities and repetition rates of the stimuli are given in the figure captions (for further details see also Stieve *et al.*, 1994).

The physiological saline contained (mM): 481 NaCl, 10 KCl, 25 $MgCl_2$, 30 $MgSO_4$, 10 $CaCl_2$, 10 NaOH, 10 HEPES (pH 7.5). Test solutions differed from physiological saline in their ionic composition. In nominally Ca-free saline, $CaCl_2$ was replaced by equal amounts of $MgCl_2$. Lithium saline contained 481 mM LiCl instead of NaCl, and 10 mM NaOH was replaced by 10 mM KOH. The superfusate was exchanged to 90% within 3 minutes.

Data analysis

Data were analyzed according to the procedure described by Stieve *et al.* (1994). The light-induced intensity change of the photometric beam after

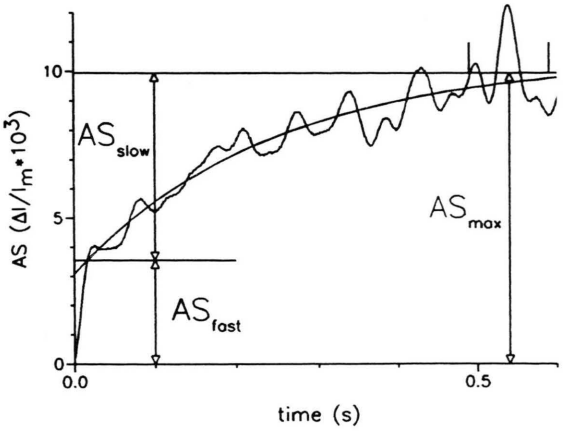

Fig. 1. Single arsenazo signal evoked by a strong flash in a cell bathed in physiological saline (30 Hz low pass filtered). Time 0 indicates the start of the arsenazo signal. The exponential of the slow rising phase is drawn. The intersection of this slow exponential with the fast increasing phase of the signal determines the amplitude of the fast phase AS_{fast}; AS_{slow}, the amplitude of the slow phase, is the peak amplitude AS_{max} minus AS_{fast}. AS_{max} is averaged from 100 values around the highest point (range between two vertical lines).

passing through the photoreceptor (ΔI) was normalized to the intensity before the stimulus (I_m). For the complete mathematical description of the arsenazo signal we improved the signal-to-noise ratio by averaging 2–4 signals and by 100 Hz low pass filter. Since the latency varied from signal to signal the start of each signal was determined by eye and set to time zero. The maximum amplitude (AS_{max}; Fig. 1) was calculated by averaging 100 points around the highest value of the 30 Hz low pass filtered digitized single trace. The time-to-peak is the time from the onset of the light stimulus to the highest point of the signal. Each trace was visually controlled and parameters corrected if necessary.

Mathematical description of the arsenazo signal

The mathematical procedure is derived from the "curve-peeling" method (Jacquez, 1972). Stieve *et al.* (1994) already analyzed the decay of the arsenazo signal by a similar graphical method. They determined the fit parameters by eye, while we determined all parameters by computer calculation. Using this method we estimated the minimal number of exponentials by which an arsenazo signal can be described. The procedure is illustrated in Fig. 3A-C. First the decay of the arsenazo signal was analyzed: In a semi-logarithmic plot with linear time axis exponential kinetics can be fitted by linear regression. The corresponding exponential function was calculated, and its difference to the arsenazo signal was fitted by linear regression, too. Thus, this procedure resulted in two exponentials describing the decay of the signal. For the analysis of the signal rise, a new curve AS' was created by subtracting each point of the original signal from the peak amplitude ($AS'=AS_{max}-AS$). This new curve was fitted as described for the decay. Finally the sum of the four exponentials was calculated using the equation

$$AS = -Ae^{(-t/\tau_1)}-Be^{(-t/\tau_2)}+Ce^{(-t/\tau_3)}+De^{(-t/\tau_4)} \quad (1)$$

The time constants of the signal rise are τ_1 and τ_2, those of the decay are τ_3 and τ_4, and A-D are scaling factors. These time constants and scaling factors were used as starting parameters for a least square fit. A complete curve fit is shown as an example in Fig. 3C. In some figures only the fit of the slow phase in the rise of the arsenazo signal is drawn to facilitate the visual discrimination between the two phases in the rise of the signal. This fit was also used to graphically determine the amplitudes of both rising phases (Fig. 1). These amplitudes cannot be calculated from the scaling factors because the parameters in the rising phase and the decay influence each other. The intersection of this fit with the fast rising phase of the signal indicates the amplitude AS_{fast} of this fast phase. The amplitude AS_{slow} of the slow rising phase was determined by subtracting AS_{fast} from AS_{max}.

Results

Graphical determination of exponential functions in the arsenazo signal

Fig. 2 shows an arsenazo signal and the corresponding receptor current trace caused by strong illumination. The receptor current in this case shows two peaks which have been suggested to be based on the opening of two different types of ion channels (Nagy, 1993; Nagy, Contzen and Stieve, 1993). The rise of the arsenazo signal indicates an increase in cytosolic free calcium concentration. The arsenazo signal reaches its maximum when the receptor current has already decayed to 10–20% of its peak amplitude. In eight cells arsenazo

signals were analyzed by determining underlying exponentials as illustrated in Fig. 3. More than one exponential was necessary to fit the rising phase as well as the decay of the arsenazo signal when the cell was superfused with physiological saline. Two phases with different slopes, i.e. different time constants could be distinguished in the rise of the arsenazo signal. The initial fast phase had an average time constant τ_1 of 0.07 ± 0.016 s (mean ± SEM, n=8), the slow further increase had a τ_2 of 0.61 ± 0.093 s. In each signal the time constant of the slow phase was at least three times larger compared to that of the fast phase. The decay consisted of two phases as well. The fast phase of the decay had a time constant τ_3 of 0.68 ± 0.093 s and the slow decline had a τ_4 of 9 ± 2 s. Thus, at least four exponentials are necessary for a satisfactory mathematical description of the arsenazo signal according to equation 1 (see Methods). Since the decay of the arsenazo signal has been studied in

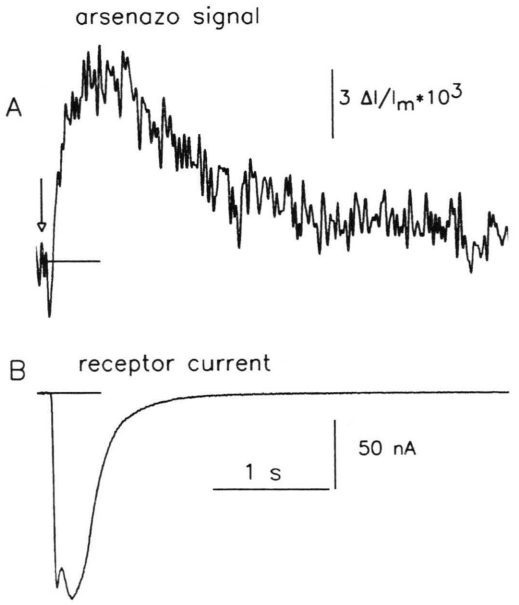

Fig. 2. Responses of the *Limulus* photoreceptor to a strong flash (5×10^{14} photons/cm^2) indicated by the arrow. The nerve was bathed in physiological saline. The cell was clamped to the pre-stimulus-membrane potential (-82 mV), 0.5 s before each flash. Flashes were delivered every 180 s. Horizontal lines indicate zero. A, average of 3 arsenazo signals (30 Hz low pass filtered). The rise of the signal indicates increasing free cytosolic calcium concentration in relative units $\Delta I/I_m$; I_m: transmission of the unstimulated cell, ΔI: light-stimulated transient transmission change. B, simultaneously measured receptor current trace.

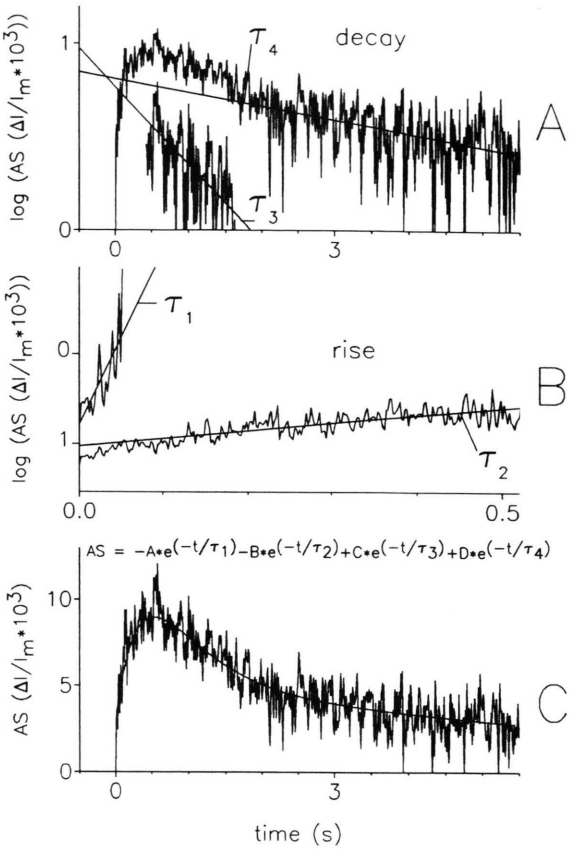

Fig. 3. Mathematical description of an arsenazo signal (average of 4) measured in physiological saline (same experiment as in Fig. 2). Time 0 indicates the start of the signal (see Methods). A, logarithmic arsenazo signal, plotted versus time. The linear part of the decay was fitted by linear regression. The slope of the regression line was used to calculate the time constant τ_4 of the corresponding exponential. The difference between the beginning of the decay and the regression line was plotted and fitted by linear regression, too. From this regression line the time constant τ_3 was calculated. The sum of the two exponentials with different time constants τ_3 and τ_4 described the decay of the arsenazo signal. B, to get exponential kinetics, the rise of the arsenazo signal was subtracted from the maximum amplitude and plotted in semi-logarithmic scale. Note that the y-axis is reversed. The time axis is linear with expanded scale. A long part of the rise appears linear and was fitted by linear regression. The time constant τ_2 of the corresponding exponential was calculated, and the difference between the start of the signal and the regression line was plotted. It could be fitted by linear regression, too, and the corresponding time constant τ_1 of the exponential was calculated. Thus, the sum of four exponentials is necessary for a complete description of the arsenazo signal. C, linear plot of the arsenazo signal and the sum of four exponentials fitted to the signal.

Cell	A	B	C	D
1	12.0	65.2	74.4	4.2
	13.8	142.1	148.1	7.6
2	6.1	188.4	185.9	8.7
	5.2	258.5	258.8	4.3
	1.7	39.4	34.0	7.5
3	8.5	230.9	230.5	9.9
4	11.9	104.8	104.1	13.3
5	3.5	482.6	480.7	5.1
6	10.9	24.7	31.7	3.4
7	5.3	84.7	86.6	4.0
8	3.1	17.1	16.8	3.5
mean ± SEM	7 ± 1.5	149 ± 48.7	150 ± 48.4	6.5 ± 1.1
Cell	τ_1 (s)	τ_2 (s)	τ_3 (s)	τ_4 (s)
1	0.098	0.9	0.9	9.7
	0.102	0.63	0.65	8.5
2	0.018	0.42	0.43	2.7
	0.019	0.52	0.55	10.0
	0.031	0.21	0.26	2.7
3	0.092	0.9	0.93	18.6
4	0.09	0.78	0.75	3.1
5	0.018	0.44	0.44	5.8
6	0.1	0.52	0.52	7.6
7	0.13	1.02	1.12	17.9
8	0.013	0.31	0.89	10.1
mean ± SEM	0.07 ± 0.016	0.61 ± 0.093	0.68 ± 0.093	9 ± 2.0

Table I. Fit parameters of the complete arsenazo signal. Parameters were calculated by least square fit from 8 cells (indicated by numbers). For cells 1 and 2, parameters of two or three signals are shown to illustrate their variability. A-D are scaling factors, τ_1-τ_4 (s) are time constants according to Eqn. (1).

detail elsewhere (Deckert and Stieve, 1991; Stieve *et al.*, 1994), the present paper is focused on the investigation of the rising phase.

Effects of membrane voltage on the arsenazo signal rise

To determine the effect of the membrane voltage on the arsenazo signal, cells were clamped to voltages between -80 mV and +30 mV (4 cells). The amplitude of the single arsenazo signals AS_{max} was dependent on the voltage in one cell (Pearson's correlation coefficient r = -0.97). In three other cells voltage dependence was not detected. A more detailed analysis, however, showed that the amplitude of the slow phase AS_{slow} was the smaller the more positive the membrane voltage was in all four cells (Figs. 4,5A; Tab. II). From an amplitude-versus-membrane potential plot we determined the reversal potential of the slow rising phase by extrapolation. The reversal potential of AS_{slow} derived from linear regression of each of four cells was +196 ± 54.7 mV (mean ± SEM, 4 cells). Pearsons correlation coefficient for each experiment was -0.8 ± 0.1 (mean ± SEM; n=4). In contrast, the fast phase of the rise was not detectably influenced by the membrane voltage (Fig. 5B and Tab. II).

Effects of reduced external calcium

To investigate the effect of a reduced driving force for calcium on the photoresponse, we superfused the preparation with saline containing no added calcium. In this nominally Ca-free saline the preparation stayed for about 45 min in full darkness. Subsequent repetitive illumination caused an amplitude reduction of the fast rising phase in single arsenazo signals (Fig. 6). The receptor current was increased and prolonged at the same time. After about 10–20 cycles of illumination the fast rising phase was no longer detectable, and the arsenazo signal consisted only of the slow rising phase. The clamped membrane voltage was varied in Ca-free saline when the fast rising phase was no longer detectable (Fig. 7). This procedure allowed

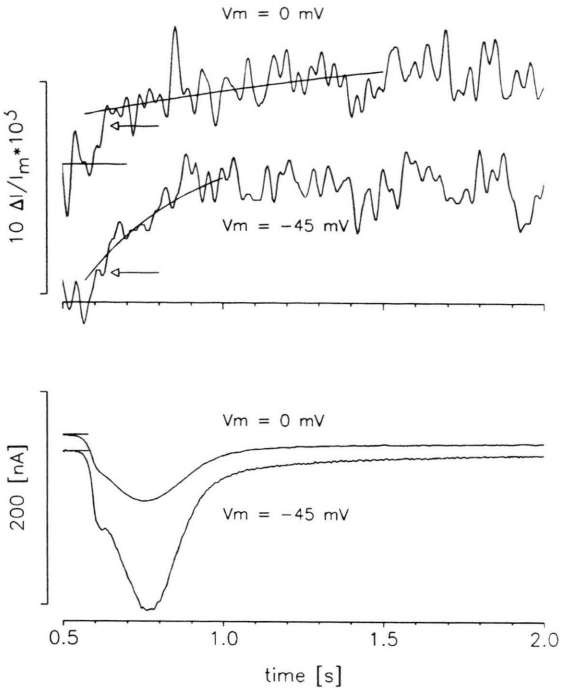

Fig. 4. Rise of arsenazo signals and receptor currents at different membrane voltages (V_m). Voltage clamp was turned on 0.5 s before the light flash. The nerve was bathed in physiological saline and illuminated (5×10^{14} photons/cm^2) every 240 s. Horizontal lines indicate zero. To prevent overlap the signals are not shown on top of each other. Upper graph: single arsenazo signals (30 Hz low pass filtered). The arsenazo signals start with a fast phase indicated by horizontal arrows. The fit of the slow phase is plotted within each signal. Lower graph: receptor currents.

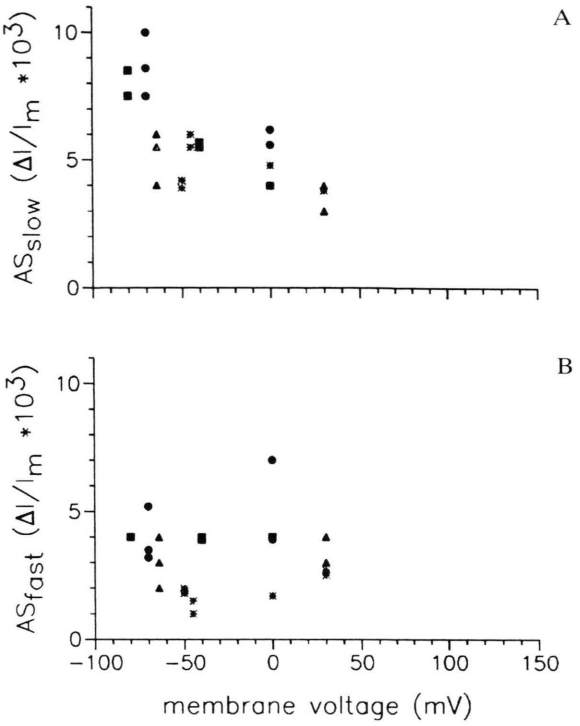

Fig. 5. Amplitudes of the rising phases of the arsenazo signal in dependence of the membrane voltage. Each data point was calculated from one response (30 Hz low pass filtered). Different symbols indicate different cells (4 cells). A, amplitude of the slow rising phase AS_{slow}; correlation coefficient r = -0.8 ± 0.19. B, amplitude of the fast rising phase AS_{fast}; r = 0.4 ± 0.4.

to investigate the influence of membrane voltage on the amplitude of the slow rising phase only. The amplitude of the slow rising phase was the smaller the more positive the membrane voltage was (Fig. 8). The reversal potential of the amplitude of the slow rising phase was determined to be +20 ± 5 mV (mean ± SEM, 2 cells).

Effects of lithium on the arsenazo signal

The increase in free cytosolic calcium could be at least partly due to the Na-Ca exchanger in the plasma membrane working in reverse direction and transporting calcium into the cell. In order to test this possibility we completely replaced sodium by lithium in the bathing medium. In *Limulus* ventral photoreceptors lithium has been shown to inhibit the sodium-driven calcium export because it is no functional substitute for sodium. In addition, it seems to block the uptake of calcium into internal stores (Stieve *et al.*, 1994). In lithium saline both decay phases of the arsenazo signal were slowed down (4 cells, Fig. 9). This result agrees with previous results of Stieve *et al.* (1994) and indicates that the short stay in lithium saline was sufficient to exert the influence of lithium on the Na-Ca exchanger and the calcium uptake into the internal stores.

The rise of the arsenazo signal was affected by lithium, too. The amplitude AS_{fast} of the fast phase was drastically reduced, and mainly due to this reduction, the amplitude AS_{max} of the signal was diminished on the average to 50%. The slow phase in the rise, however, was not detectably influenced.

Table II. Parameters of the arsenazo signal in dependence of the membrane voltage (V_m in millivolts; same experiments as in Fig. 5). The experiments were carried out with the two electrode voltage clamp technique. Data are shown for each cell indicated by numbers. Cell numbers are corresponding to symbols in Figs. 7,8: Cell 1 = circles, 2 = squares, 3 = triangles, 4 = stars. AS_{max}, AS_{fast} and AS_{slow} are shown in $\Delta I/I_m \times 10^3$.

		Membrane voltage							
Cell	Parameter	−80	−70	−65	−50	−45	−40	0	+30
1	AS_{max}		13.8					12.6	
			13.5					10.9	
			10.7						
	AS_{fast}		5.2					7.0	
			3.5					3.9	
			3.2						
	AS_{slow}		8.6					5.6	
			10.0					6.2	
			7.5						
2	AS_{max}	12.5					9.5	8.0	
		11.5					9.6		
	AS_{fast}	4.0					4.0	4.0	
		4.0					3.9		
	AS_{slow}	8.5					5.5	4.0	
		7.5					5.7		
3	AS_{max}			8.0					8.0
				7.0					6.0
				9.5					
	AS_{fast}			2.0					4.0
				3.0					3.0
				4.0					
	AS_{slow}			6.0					4.0
				4.0					3.0
				5.5					
4	AS_{max}				6.0	7.5		6.5	6.3
					5.9	6.5		5.7	6.5
	AS_{fast}				1.8	1.5		1.7	2.5
					2.0	1.0		1.7	2.7
	AS_{slow}				4.2	6.0		4.8	3.8
					3.9	5.5		4.0	3.8

Discussion

The sum of four exponentials sufficiently describes the complete light-evoked signal of the calcium indicator arsenazo III. Since the decay phase of this signal has been studied in detail by Deckert and Stieve (1991) and Stieve *et al.* (1994), we here focus on the characterization of the signal rise. With the graphical method we employed, a single exponential was not sufficient to fit the entire rising phase. The arsenazo signal starts with a fast increase which was independent of the membrane voltage. At the end of this fast phase the signal further increases slowly, a process that has been described here for the first time. This slow phase is voltage-dependent. After removal of calcium in the superfusate, the fast rising phase of the arsenazo signal was drastically decreased due to repetitive illumination. The slow rising phase was voltage-dependent in Ca-free saline and its reversal potential, extrapolated from AS_{slow}-versus-membrane voltage plots, was shifted to a more negative value compared to physiological saline. The complete replacement of sodium by lithium in the superfusate strongly reduced the fast rising phase but did not detectably influence the slow increase in the arsenazo signal. From these experiments we conclude that the fast rising phase in the signal is caused by calcium release from internal stores. The slow phase, on the other hand, could be due to calcium influx from the extracellular space via light-activated channels in the plasma membrane.

The occurrence of two phases in a calcium indicator signal does not necessarily reflect different phases in the rise of free cytosolic calcium in the

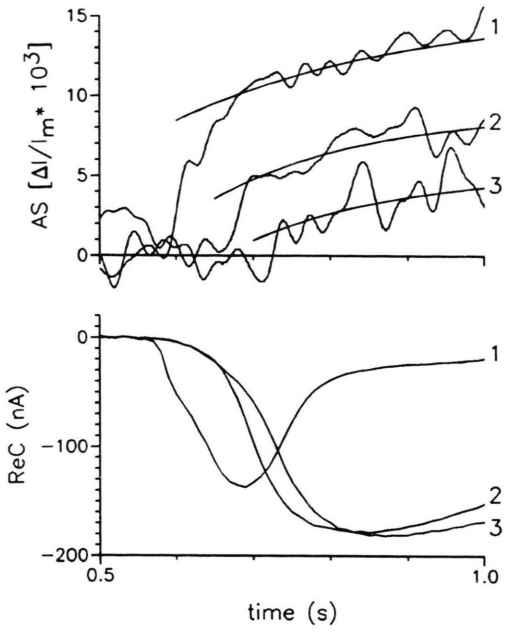

Fig. 6. Arsenazo signal and receptor current before and after removal of external calcium. Responses recorded in physiological saline (1). Records in Ca-free saline after 6 cycles of illumination (2) and after 18 cycles of illumination (3). Upper graph: single arsenazo signals (30 Hz low pass filtered); lower graph: receptor current.

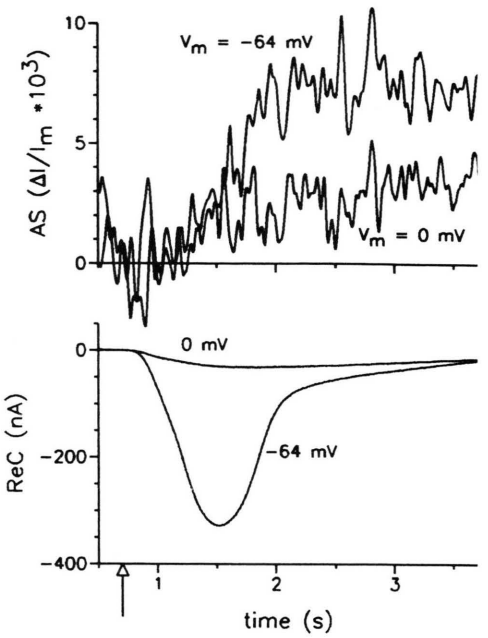

Fig. 7. Arsenazo signal and receptor current in Ca-free saline at different membrane voltages (V_m). Signals were recorded when the fast rising phase in the arsenazo signal was no longer detectable, i.e. the maximum amplitude of the signal is identical with the amplitude of the slow rising phase. Upper traces: single arsenazo signals (30 Hz low pass filtered); lower traces: receptor current. The stimulating flash is indicated by an arrow.

cell. Instead, it could be due to methodical artifacts based e.g. on binding kinetics of the calcium indicator. Clemen, Rabl and Neumann (1988) described a fast arsenazo-calcium binding mode *in vitro* (time constant: 4–20 ms) when the calcium concentration was below 100 μM. This could underlie the fast phase in the arsenazo signal. Calcium concentrations above 100 μM caused an additional slow reaction mode (time constant: 100–600 ms; Clemen *et al.*, 1988), which could be responsible for the slow phase in the arsenazo signal rise. Alternatively, light could have induced high local calcium concentrations (fast phase) saturating the indicator. A subsequent diffusion of calcium into regions with unsaturated arsenazo could have caused the slow phase. In neither case the membrane voltage should affect arsenazo binding kinetics. However, our experiments clearly reveal a voltage dependence of the slow phase, while the fast phase was independent of the membrane voltage. The fast rising phase was influenced by the experimental conditions, too. After removal of external calcium the fast rising phase was drastically reduced following repetitive illumi-

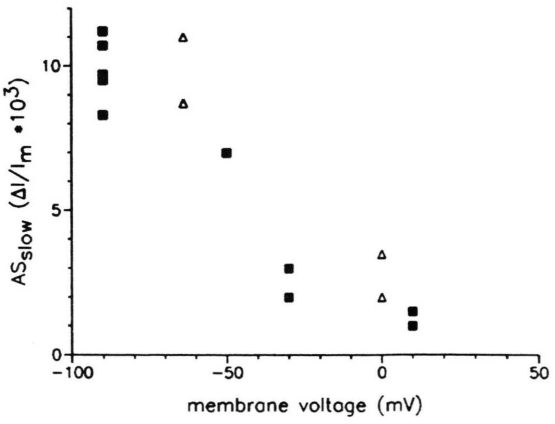

Fig. 8. The amplitude of the slow rising phase is plotted versus the membrane voltage. Different symbols indicate different cells as in Fig. 5. Each data point was calculated from a single arsenazo signal (30 Hz low pass filtered). Pearson's correlation coefficient from each experiment r = -0.964 ± 0.0014 (mean ± SD; n = 2).

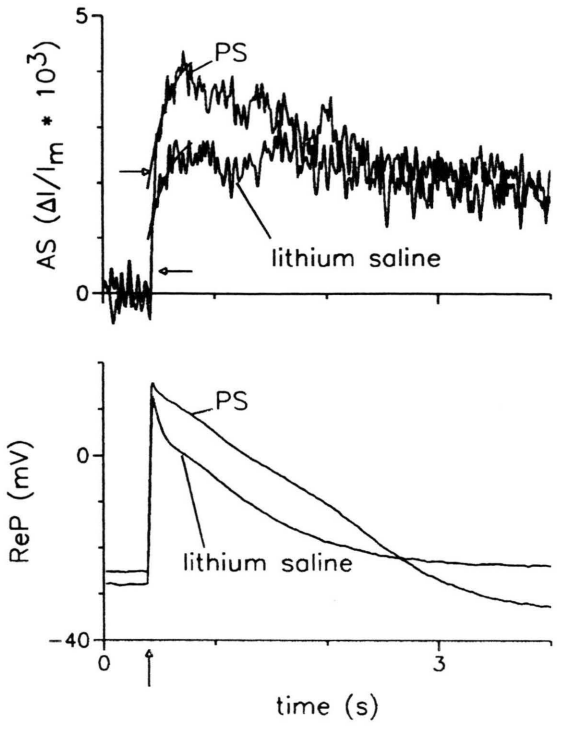

Fig. 9. Arsenazo signals (each averaged from 4 signals) and receptor potential (ReP) before and 12 min after exchange of the superfusate to lithium saline. A light stimulus (9×10^{14} photons/cm^2, vertical arrow) was applied every 60 s. The fit of the slow exponential in the rise of the signals is plotted. The fast phase in the rise is marked by horizontal arrows. PS: physiological saline.

nation (for detail see Results). These findings exclude the possibility that arsenazo binding kinetics or local differences in cytosolic calcium concentration are responsible for the two phases in the signal rise. Our results strongly suggest that the two phases indeed reflect two different cellular processes leading to the light-stimulated increase in $[Ca^{2+}]_i$.

The slow phase of the rise in $[Ca^{2+}]_i$ after a flash was increasingly accelerated the more negative the membrane voltage was. The amplitude of the slow rising phase increased in parallel. This indicates that light stimulates an influx of calcium across the plasma membrane. Light-evoked calcium influx dependent of the membrane voltage has been proposed earlier because calcium indicator signals (aequorin, arsenazo III) were reduced in depolarized cells (Brown and Blinks, 1974; Ivens and Stieve, 1984). Deckert and Stieve (1991) ascribed this to cell desensitization due to the reverse action of the Na-Ca exchanger which had been activated by pre-stimulus membrane depolarization under voltage clamp conditions. They found no significant effect of voltage changes on the amplitude of the arsenazo signal when they clamped the membrane voltage to the desired value only five hundred milliseconds before the light stimulus. In most of our experiments, the amplitude of the overall arsenazo signal was not voltage dependent either. Only the detailed analysis of the two rising phases clearly shows the voltage dependence of the slow rising phase. We reduced the driving force for calcium influx by removal of external calcium. With 10 mM calcium in the superfusate and assuming a $[Ca^{2+}]_i$ of 0.4–0.5 μM (Ukhanov, Flores, Hsiao, Mohapatra, Pitts and Payne, 1995), the Nernst potential in physiological saline is about +125 to +128 mV. Ukhanov *et al.* (1995) found a reduction of $[Ca^{2+}]_i$ to about 0.2 μM $[Ca^{2+}]_i$ when the nerve was bathed in Ca-free saline with 1 mM EGTA added. Due to residual calcium the $[Ca^{2+}]$ in the nominally Ca-free superfusate was estimated to be about 40 μM (Maaz and Stieve, 1980). With 40 μM calcium in the superfusate and 0.2 μM $[Ca^{2+}]_i$, the Nernst potential is about +64 mV. Since we used a higher external calcium concentration than Ukhanov *et al.* (1995), we expected a larger $[Ca^{2+}]_i$ than 0.2 μM. Therefore the calculated Nernst potential of +64 mV indicates an upper limit value. In any case the reversal potential for calcium influx would be shifted to more negative values in Ca-free saline compared to data measured in physiological saline. Indeed, the reversal potential of the slow rising phase in the arsenazo signal was about +196 mV in physiological saline and shifted in negative direction in Ca-free saline (+20 mV).

Our experiments with lithium saline were designed under the assumption that lithium at least partially inhibits the activity of the Na-Ca exchanger because it is not transported as a substitute for sodium (O'Day and Gray-Keller, 1989; O'Day *et al.*, 1991; Stieve *et al.*, 1994). The slow rising phase in the arsenazo signal was unaffected in lithium-containing saline. This finding argues against the notion that the slow phase could be caused by calcium influx via the Na-Ca exchanger working in reverse mode. In addition, τ_2, the time constant of the slow rising phase, is about one or-

der of magnitude faster than τ_4, the time constant of the slow decay phase which is supposed to be due to the extrusion of calcium *via* Na-Ca exchanger (Deckert and Stieve, 1991). This too, suggests that the two phases are caused by different cellular processes. On the other hand, we cannot rule out the unlikely possibility that the intracellular concentration of sodium remains high despite removal of external sodium. The outward gradient of sodium across the plasma membrane could then cause an electrogenic extrusion of sodium against calcium via the Na-Ca exchanger if the experiments are not done under voltage clamp conditions, as was the case in this part of our measurements.

Another possible way for calcium ions to flow from the external space into the cell is through voltage-activated channels in the plasma membrane as has been proposed by Lisman, Fain and O'Day (1982) for *Limulus* ventral photoreceptors. In this case we would have had to expect an accelerated calcium influx with membrane voltages more positive than the pre-stimulus membrane potential. The contrary was true in our study, since the slope of the slow rising phase was the steeper the more negative the membrane potential was, and the amplitude was increased in parallel. We therefore assume that calcium enters the cell via light-activated ion channels in the plasma membrane.

The fast phase in the rise of the arsenazo signal was independent of the membrane voltage and may be attributed to release of calcium from internal stores. This seems to be the main source of the light-stimulated increase in cytosolic free calcium in *Limulus* under physiological conditions (Brown and Blinks, 1974; Corson and Fein, 1987; Payne, Walz, Levy and Fein, 1988; Deckert and Stieve, 1991; for review see Fein and Payne, 1989). Payne and Flores (1992) measured IP_3-evoked calcium release in *Limulus* ventral photoreceptors by means of aequorin while the cells were bathed in calcium-free saline. The maximum of the aequorin signals was reached after 100–200 ms (at 11 °C as well as at 21 °C; their Fig. 3). This is comparable to the time course of the fast rising phase (time constant: 70 ± 16 ms; mean ± SEM) of the arsenazo signal in our experiments. After removal of external calcium in full darkness repetitive illumination led to a drastical exhaustion of the fast rising phase. This agrees with the finding that internal stores can only be depleted in Ca-free saline when the cells are illuminated (Maaz and Stieve, 1980; Bolsover and Brown, 1985).

Lithium reduced the fast phase in the rise of the arsenazo signal, indicating that the IP_3-sensitive calcium release from internal stores is impaired. According to the inositol depletion hypothesis, which is based on studies from different preparations (e.g. rat brain; Allison and Stewart, 1971), lithium reduces the intracellular concentration of inositol. As a consequence, the enzymatic production of IP_3 is reduced (for review see Berridge, Downes and Hanley, 1989). In *Limulus* ventral photoreceptors lithium reduced and slowed down the electrical response, which recovered only after extracellular application of inositol (O'Day, Johnson and Baumgard, 1991). In addition, lithium seems to inhibit the uptake of calcium into the internal stores (Stieve *et al.*, 1994). This could eventually lead to calcium depletion of the stores and thus reduction of the fast phase. Besides the well established IP_3-dependent Ca-release, it cannot be ruled out that calcium-induced calcium release might cause part of the arsenazo signal. This

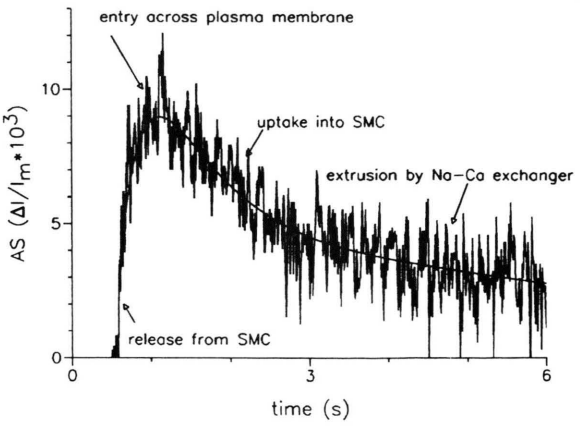

Fig. 10. Arsenazo signal as in Fig. 3C with additional interpretation of the calcium regulating mechanisms that underlie the four detectable phases. Time constants for the different phases have been derived from the fit of the arsenazo signal. They are 0.01 s (fast rising phase), 0.47 s (slow rising phase and fast decay phase) and 7.5 s (slow decay phase). The contribution of each rising phase has been calculated as the ratio of each exponential to the entire signal rise (AS_{fast}/AS_{max} = 36%; AS_{slow}/AS_{max} = 64% see Fig. 1). The contributions of the decay phases were calculated accordingly. They were 59% for the fast decay and 41% for the slow decay. Submicrovillar cisternae (SMC) are the internal calcium stores.

Ca-release mechanism was found in photoreceptors of the honeybee for the first time (Walz, Baumann, Zimmermann and Ciriacy-Wantrup, 1995).

O'Day and Keller (1989) first demonstrated two phases in the decrease in intracellular calcium. Deckert and Stieve (1991) proposed that the fast phase in the decay of $[Ca^{2+}]_i$ was caused by the uptake of calcium into submicrovillar cisternae. The slow phase is electrogenic and was explained by the extrusion of calcium via a Na-Ca exchanger. The decay phase of the arsenazo signal in our experiments also had two phases with time constants similar to those already described (Deckert and Stieve, 1991; Stieve *et al.,* 1994). The complete description of an arsenazo signal needs the sum of at least four exponentials. As is summarized in Fig. 10, we therefore conclude that there are at least four cellular processes which prominently regulate the light-stimulated change in $[Ca^{2+}]_i$, namely release from internal stores, influx across the plasma membrane, uptake into the internal stores, and extrusion *via* the Na-Ca exchanger.

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