Cytosolic Calcium Transient and Light Response in *Limulus* Ventral Nerve Photoreceptors: Supralinearity in Response-Intensity Curves

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Light-evoked intracellular electrical responses were measured simultaneously with the changes in intracellular free Ca^{2+} or Sr^{2+} concentration by means of arsenazo III. The response-versus-stimulus intensity characteristics was measured in two states of relative light adaptation in strontium-containing saline (10 mM Sr^{2+}; 0.25 mM Ca^{2+}). Supralinear parts of the curves with slopes > 1 were found for the receptor current amplitude and the current-time integral as well as for the amplitude of the arsenazo signal within at least 0.3 log units of identical stimulus intensity range. Maximum slopes of response-versus-intensity curves are on average approximately 1.7 for the receptor current and the arsenazo signal in both adaptational states. The rise in time of the arsenazo signal evoked by a light flash shows two phases. In strontium saline we observe a fast increase at the onset of the arsenazo signal which is detectable within the first 20–100 ms of the signal rise and a slow further increase to the maximum, with a duration of 0.7–6 s. In the detailed analysis of both phases we observed that the amplitude of each phase increases supralinear, i.e. disproportionate to the stimulus intensity. The fast rising phase of the arsenazo signal is not visibly voltage-dependent. This fast rising phase might be a monitor of an IP_3-sensitive calcium or strontium release. Since the amplitude of the fast rising phase increases supralinear with the stimulus intensity, we propose the interpretation that more than one IP_3-molecule must bind to the IP_3-receptor to open this channel of the intracellular calcium store. Only the slow rising phase is voltage-dependent. The kinetic of this slow rising phase was accelerated with more negative membrane potentials. This predominant slow phase correlates with the receptor current-time integral. Therefore the slow rising phase of the arsenazo signal might be a monitor for influx of calcium or strontium through light activated ion channels in the plasma membrane. The supralinearity of the amplitude of the slow rising phase might indicate the supralinear increase in calcium or strontium, which is carried by the receptor current. Additionally, we calculated the fraction of strontium and calcium ions of the total receptor current. When calcium was replaced by strontium in the superfusate about 3–30% of the receptor current are carried by strontium and calcium ions. In physiological saline calcium contributes about 1–1.5% to the receptor current.

Introduction

In *Limulus* ventral photoreceptors light causes a transient depolarisation of the plasma membrane. Inositol 1,4,5-trisphosphate (IP_3) and calcium are mediators of the phototransduction in *Limulus* ventral photoreceptors. Calcium is released from IP_3-sensitive internal calcium stores (Brown and Rubin, 1984; Payne, Corson, Fein and Berridge, 1986b). Besides IP_3-induced calcium release the existence of a calcium-induced calcium release was documented in photoreceptors of the honeybee (Walz *et al.,* 1995). The extracellular space is an additional source for the light-induced rise in cytosolic calcium concentration in photoreceptors of barnacle (Brown and Blinks, 1974; Werner *et al.,* 1992), honeybee (Minke and Tsacopoulos, 1986; Ziegler and Walz, 1989), *Lima* and *Pecten* (Gomez and Nasi, 1996) and fly (Hardie, 1991, Ranganathan *et al.,* 1991), while in *Limulus* a light-induced influx of calcium is controversially discussed (Brown and Mote, 1974; Brown and Blinks, 1974; Ivens and Stieve, 1984; Deckert and Stieve, 1991; for review see Nagy, 1991). Measured with the calcium indicator arsenazo III, a fast rising phase of the calcium transient, indicating calcium release from internal stores, can be distinguished from a slow rising phase. This slow phase indicates

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calcium influx across the plasma membrane (Stommel, Dorlöchter, Rüsing and Stieve, 1996). The excitatory action of calcium in invertebrate photoreceptors is not yet fully clear (Bacigalupo, Johnson, Vergara and Lisman, 1991; Hardie, 1994; Ukhanov and Payne, 1995, Brown and Kelman, 1996). The light-stimulated rise of intracellular calcium is known to mediate light adaptation (Lisman and Brown, 1972; Lisman and Brown, 1975; Nagy and Stieve, 1983; Stieve, Bruns, and Gaube, 1984; Payne, Corson and Fein, 1986a; Stieve, Gaube and Klomfaß, 1986). In *Limulus* ventral photoreceptors it has already been shown that log-log plots of the current amplitude or the current-time integral versus the stimulus intensity show a supralinear region with a slope >1, i.e. the response increases disproportionate to the stimulus intensity (Brown and Coles, 1979; Stieve *et al.,* 1986; Stieve and Schlösser, 1989). More recently it has been reported that this supralinearity can be found only in one of three different receptor current components (Deckert, Nagy, Helrich and Stieve, 1992; Contzen and Nagy, 1996). In the present study we measured the light-stimulated electrical response simultaneously with the transient change of intracellular free Ca^{2+} or Sr^{2+} concentration. In strontium-containing saline the effect of different membrane potentials on both signals was tested, and additionally the intensity dependence of both photoresponses in two different states of light adaptation. Arsenazo indicates strontium similarly to calcium (see Materials and Methods). Since the decay of the arsenazo signal measured in strontium saline has already been described earlier (Stieve *et al.,* 1994), we mainly analysed the rising phase of the arsenazo signal which consists of two phases with different kinetics. Under the assumption, that light-activated channels, which are calcium- and strontium permeable, exist in the plasma membrane, the contribution of both cations to the receptor current has been calculated. Parts of the present study have been published in abstract form (Stieve and Stommel, 1992; Stommel *et al.,* 1994).

Materials and Methods

The experimental technique was similar to that described by Deckert and Stieve (1991). Ventral nerves were dissected from male *Limulus polyphemus* obtained from the Marine Biological Laboratories (Woods Hole, MA). After desheathing, the nerve was fastened to a silicone rubber block and kept in organ culture medium (Kass and Renninger, 1988) for 3–48 h at 6 °C. The nerve was treated with collagenase (2 mg/ml physiological saline) for 10 minutes and placed in a glass chamber (1.5 ml volume), which was perfused at a rate of 1 ml saline per minute at constant temperature (15 °C). The glass micropipette used for intracellular recording and injection was filled with 0.5 M KCl, and the tip of the micropipette contained 20 mM arsenazo III in 0.5 M KCl. The electrode resistance was between 4 and 15 MΩ. Electrical recordings were done with a single electrode voltage clamp device (Biologic, France). Because the clamping was not perfect, the receptor current was accompanied by an undesired transient depolarisation of the plasma membrane with a peak depolarisation proportional to the peak amplitude of the current (0.05–0.1 mV/nA). Arsenazo was injected into the cell with 1–4 bar pressure. A photometric beam (645 nm half width ± 9 nm; intensity of the photometric beam after passing the unstimulated cell I_m = 0.1–1 mW/cm^2; diameter approximately 125 μm) focused onto the cell decreased the light sensitivity of the photoreceptor by a factor of 10–100 in our experiments with strontium saline (composition see below). This measuring light was permanentely switched on. An intracellular change in the concentration of free Ca^{2+} or Sr^{2+} leads to a transient transmission change of the injected cell termed arsenazo signal, which was monitored by a photomultiplier. The sensitivity of the indicator dye for strontium (K_D= 2.5 μM) is similar to that for calcium (K_D=0.74 μM) (Kendrick, Ratzlaff and Blaustein, 1977). 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). Strontium saline contained (mM): 481 NaCl, 10 KCl, 45 $MgCl_2$, 10 $SrCl_2$, 10 NaOH, 10 HEPES (pH 7.5). Strontium saline with 0.25 mM Ca contained (mM): 481 NaCl, 10 KCl, 44.75 $MgCl_2$, 0.25 $CaCl_2$, 10 $SrCl_2$, 10 NaOH, 10 HEPES (pH 7.5). The superfusate could be exchanged to 90% within 3 minutes. The cells were stimulated with test flashes (515 ± 70 nm, wide band filter; 2 ms half width) alternating with a global conditioning stimulus applied with a halogen lamp (515 nm maximum, wide band filter;

0.06–2 s duration). The light intensity was reduced by neutral density filters.

Data analysis

Electrical responses and arsenazo signals were digitized at 1 kHz and stored by a computer (for details see Deckert and Stieve, 1991). Data were analysed according to the procedure described by Stieve and Benner (1992). J_{max} is the peak amplitude of the receptor current. The receptor current-time integral is calculated from the time when the signal exceeds twice the average noise before the light stimulus to the time when the signal decreases below this level. Arsenazo signals are given in relative units ($\Delta I/I_m$; ΔI: transmission change after light stimulation). The recorded arsenazo signal (AS) was low pass filtered (15 Hz) without visible changes of the kinetics (Deckert and Stieve, 1991) before the parameters were calculated. The peak amplitude (AS_{max}) is calculated by averaging the values of 100 ms around the largest point identified by the program. The time-to-peak is the time from the begin of the light stimulus to the peak value of the arsenazo signal. Each trace was visually controlled and parameters corrected if necessary. The kinetics of the arsenazo signal rise and the number of exponentials which are at least necessary to describe the rise, were calculated as described earlier (Stieve *et al.*, 1994; Stommel *et al.*, 1996). The calculation of the amplitude of the fast rising phase (AS_{fast}) and of the slow rising phase (AS_{slow}) are described in Fig. 1C.

Results

Effects after replacement of external calcium by strontium

Strontium is known to substitute for calcium to a certain degree and has been used to study cellular processes regulating the cytosolic calcium concentration in the ventral nerve photoreceptor (Payne *et al.*, 1986a; Stieve and Benner, 1992; Stieve *et al.*, 1994). After replacement of external calcium by strontium the pre-stimulus membrane potential shifts to more negative values and the duration of the electrical light response increases (Fig. 1D). In order to prevent large dark currents we measured in these experiments without voltage clamp. In the following we concentrate on the arsenazo signal (Fig. 1A-C). When calcium in the superfusate was completely replaced by strontium, the amplitude of the arsenazo signal increased 3–5 fold and the time-to-peak was prolonged (n=5 cells). This raise in the arsenazo signal was mainly due to the slow phase of the arsenazo signal which grew considerably (Fig. 1A,B). The contribution of this phase to the peak amplitude (AS_{slow}/AS_{max}) was 20–70% in physiological saline and increased to 70–90% after 30 min in strontium saline. The amplitude AS_{fast} of the fast phase, too, increased, but only slightly and transiently (Fig. 1B). After about two hours in strontium saline the fast rising phase was no longer detectable (2 cells). Indeed, mathematical analysis showed that the rise of the signal could be described by a single exponential in these experiments. This effect was reversible in physiological saline. Interestingly, when we superfused the nerve with strontium saline containing 0.25 mM Ca (composition see Materials and Methods, 10 mM Sr^{2+} and 0.25 mM Ca^{2+}), even after two hours the fast rising phase was not detectably reduced (4 cells).

Effects of the membrane voltage on the arsenazo signal

Ventral nerves were bathed in strontium saline with 0.25 mM Ca for at least 45 min before the membrane voltage dependence was tested (4 cells). Adopting the procedure of Deckert and Stieve (1991), in these experiments we voltage-clamped the cells to the desired value only 0.5 s before the light stimulus. This prevents an electrogenic driven influx of calcium via the Na-Ca exchanger before the light stimulus due to depolarisation of the cell. The resulting increase in calcium would desensitize the cell and lead to an undesired apparent voltage dependence of the arsenazo signal. The kinetics of the arsenazo signal rise was determined as described in Stieve *et al.* (1994) and Stommel *et al.* (1996). The slow rising phase was slowed down 2–4 fold with positive membrane voltage and the time-to-peak was prolonged in parallel (Fig. 2). The maximum amplitude (AS_{max}) of the arsenazo signal and the fast rising phase of the arsenazo signal were not detectably voltage-dependent. The receptor current was prolonged with positive membrane voltage in parallel to the prolongation of the arsenazo signal rise.

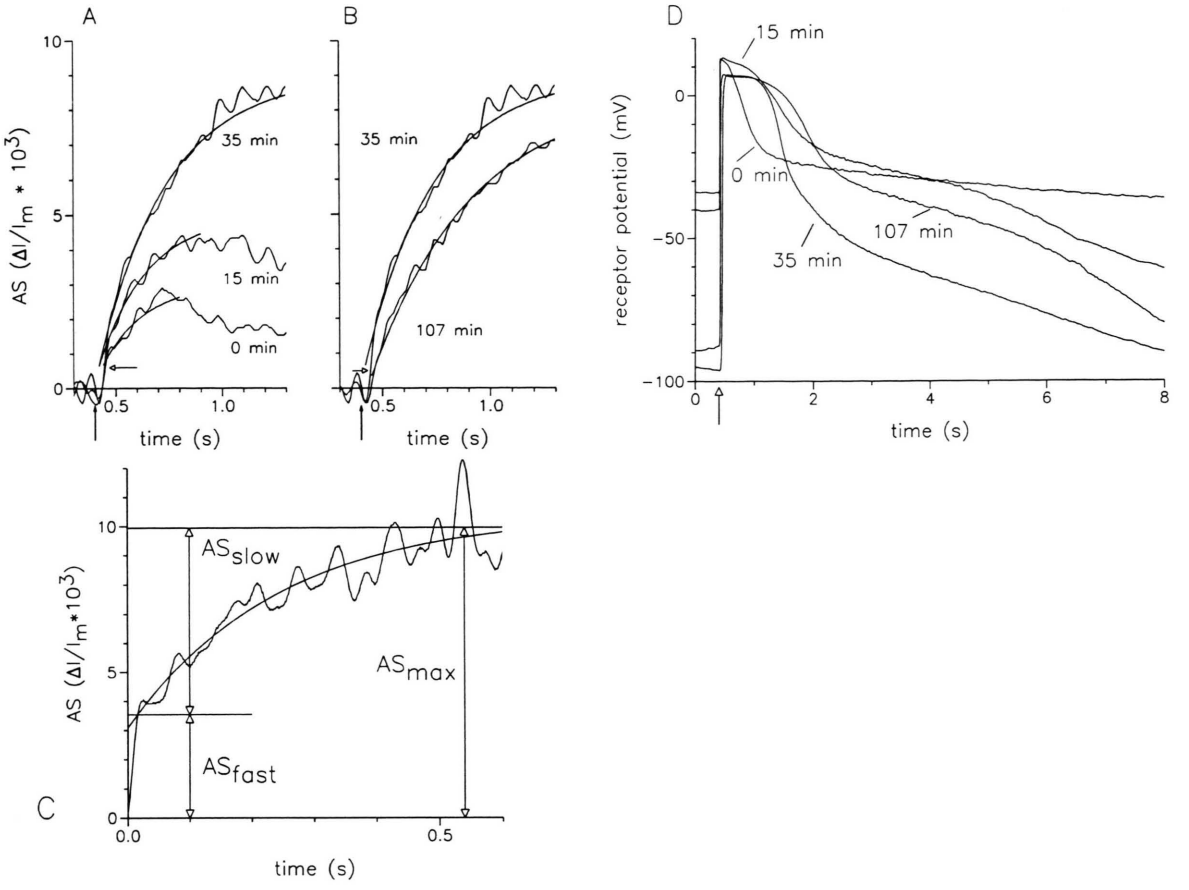

Fig. 1. Arsenazo signal (each averaged from 4 signals) (A-C) and receptor potential (D) shown at different times after exchange of the superfusate to strontium saline. Vertical arrows indicate flashes (9×10^{14} photons/cm^2) delivered every 60 s. Exponential functions were fitted to the arsenazo signal according to Stieve *et al.* (1994). The fast rising phase of the arsenazo signals are marked by horizontal arrows. A, arsenazo signals recorded at 0, 15, and 35 minutes in strontium saline. The fit of the slow phase is plotted for each signal. Typically, the main increase in the size of the arsenazo signal was reached 30–40 minutes after solution change. Note that mainly the slow phase increased in strontium saline. B, after about 2 hours in strontium saline, only the slow phase in the rise was detectable. C, calculation of the amplitude of the fast rising phase (AS_{fast}) and of the slow rising phase (AS_{slow}). Each of both rising phases could be fitted by one exponential but only the fit of the slow rising phase is shown for the signal. The intersection of this fit with the fast rising phase indicates the amplitude of the fast rising phase AS_{fast}. AS_{slow} was calculated by subtracting AS_{fast} from AS_{max}. *D*, single receptor potentials recorded at 0, 15, and 35 minutes in strontium saline.

Effects of different stimulus intensities in two states of light adaptation

The intensity dependence of the photoresponses was measured in strontium saline with 0.25 mM calcium because the arsenazo signal was larger in this saline compared to physiological saline. This enabled us to measure detectable arsenazo signals even at low stimulus intensities. As described above the 0.25 mM calcium prevented abolishing of the fast rising phase in strontium-containing saline. A constant conditioning stimulus to light-adapt the cell was followed by two test flashes of the same light intensity which was varied. The first test flash was applied to the light-adapted cell 18 s after the conditioning stimulus, and the second test flash followed 102 s later when the cell was more dark-adapted. The intensity of the conditioning stimulus was chosen to reduce the sensitivity

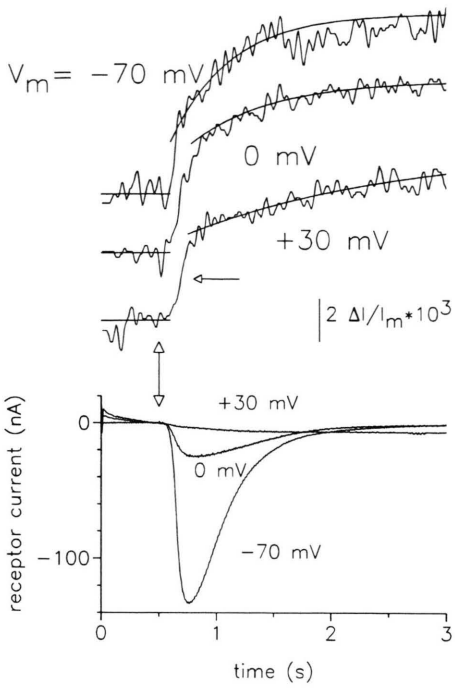

Fig. 2. Arsenazo signals and receptor current at different membrane voltages. Voltage clamp was turned on 0.5 s before the light flash. The nerve was bathed in strontium saline with 0.25 mM calcium and illuminated with 5×10^{14} photons/cm^2, (vertical arrow) every 60 s. Upper graph: the arsenazo signals (each averaged from 6 signals) start with a fast phase indicated by a horizontal arrow. The fit of the slow rising phase is plotted within each signal. To prevent overlap the signals are not shown on top of each other. Lower graph: single corresponding receptor current traces.

of the cell in such a way that the response to the first test flash was around 50% of the second one. The time between two conditioning stimuli (cycle time) was 4–6 minutes, sufficient for dark adaptation. Thus, the receptor current amplitude caused by the conditioning stimulus changed only slightly during the whole experiment. Since the cells were already light-adapted to a certain degree due to the photometric light beam, the two states of adaptation shall be termed "relative". Response-versus-stimulus intensity curves were measured from low to high test flash intensities. In these experiments the cells were voltage-clamped to the pre-stimulus membrane potential measured after about 30 min in strontium saline. At that time the pre-stimulus membrane potential was stable and the voltage clamp was turned on at a constant value (V_m= -60 ± 20 mV) throughout the subsequent experiment. Responses in both adaptational states of the cell to stimuli of different light intensities are shown in Fig. 3. The maximum amplitude of the arsenazo signal was reached when the receptor current already decayed to about 20% of the receptor current peak value. Strong light stimuli evoked arsenazo signals with a time-to-peak T_{max} of about 3–6 s, while dim light caused arsenazo signals with a time-to-peak value between 0.8 s and 1.5 s. Double logarithmic plots of the signal amplitude versus stimulus intensity are roughly sigmoidal (Fig. 4). The steepest slope was calculated between two experimental points in each curve. The average values from different experiments were 1.6–1.8 (4 cells) for J_{max} and the current-time integral, as well as for AS_{max} in both relative states of adaptation (Table IA). Supralinearity of the current-time integral and J_{max} was found within a range of about 0.3–1 log unit of stimulus intensities. This range was about 0.3–0.7 log unit for the peak amplitude of the arsenazo signal. The range of stimulus intensity where supralinearity could be observed was similar for the light-adapted cell compared to the relative dark-adapted cell. Figure 4 shows that in the relative dark-adapted cell supralinearity was evoked even

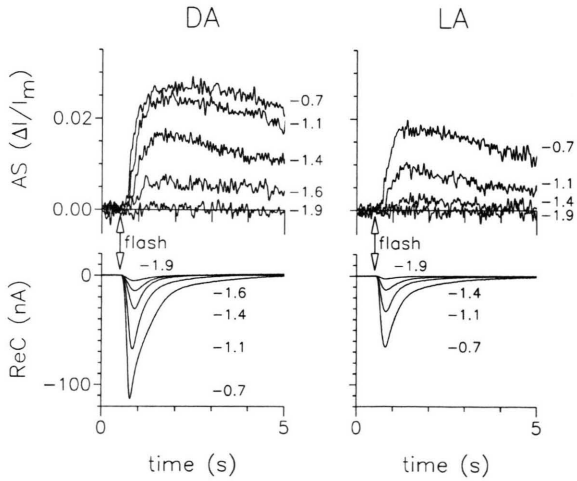

Fig. 3. Arsenazo signal (AS) and receptor current (ReC) after stimulating the photoreceptor at different light intensities. The responses are recorded in a relatively light-adapted state (LA) and a relatively dark-adapted state (DA). Stimulus intensities are presented as $\log(I/I_o)$ with $I_o=5\times10^{14}$ photons/cm^2. The nerve was bathed in strontium saline with 0.25 mM calcium for about 45 minutes before the stimulus-response-curves were measured.

Table I. Average values for the maximum slopes of response-versus-stimulus intensity curves in two states of relative light adaptation (as means ± SD) measured in strontium saline with 0.25 mM calcium. Number of experiments (n). Slopes are calculated between the two points with maximal steepness in a relative light-adapted state (LA) and a relative dark-adapted state (DA). A, experiments with injected arsenazo and the illuminating photometric beam. B, experiment with injected arsenazo but without photometric beam. C, without arsenazo but with illuminating photometric beam. D, without arsenazo and without photometric beam; same experiments as in Table II.

	Maximal slopes							
	A		B		C		D	
	With injected AIII and photometric beam (n=4)		With injected AIII (n=1)		With photometric beam (n=2)		Without AIII; without photometric beam (n=2)	
	LA	DA	LA	DA	LA	DA	LA	DA
J_{max}	1.7 ± 0.3	1.6 ± 0.3	1.4	1.4	1.4 ± 0.1	1.2 ± 0.001	1.5 ± 0.4	1.3 ± 0.003
Current-time integral	1.8 ± 0.6	1.7 ± 0.6	1.7	1.6	1.5 ± 0.4	1.2 (n=1)	1.6 ± 0.4	1.0 ± 0.2
AS_{max}	1.6 ± 0.4	1.7 ± 0.3						

with more dim light than in the light-adapted cell. At least within 0.3 log unit of stimulus intensity, supralinearity of the receptor current and the arsenazo signal was evoked correspondingly. In expanded time scale two phases can be detected in the rise of the arsenazo signals (Fig. 5A,B). We already reported that these two phases indicate two different processes which generate the increase in cytosolic free calcium (Stommel *et al.* 1996). We determined the amplitudes of the fast and the slow rising phase as illustrated in Fig. 5A and checked which of both rising phases increased supralinearly in response-versus-stimulus intensity curves. Dim light caused arsenazo signals in which only one rising phase was detectable. Because the signal was too small, our method to determine exponentials in the arsenazo signal gave no reliable information on the question whether this single rising phase is fast or slow. Strong stimuli evoked arsenazo signals with a biphasic rise. We compared single arsenazo signals at two different light intensities from the supralinear range of a response-versus-intensity curve. Viewed from low to high stimulus intensities, in all experiments supralinearity appeared when the larger arsenazo signal showed a biphasic rise and the smaller signal only one rising phase (Fig. 5A). In the example shown in Fig. 5A the size of the larger signal is mainly due to the fast rising phase. However, supralinearity was not exclusively caused by an increase in the fast phase. In one experiment supralinearity was observed although the fast phase was almost saturated (Fig. 5B). In this case apparently also the slow phase strongly contributed to the supralinearity.

Correlation of receptor current and arsenazo signal

If the arsenazo signal were an indicator for calcium or strontium influx through light-activated ion channels, its amplitude should correlate with the current-time integral. Indeed, the maximum amplitude of the arsenazo signal is correlated with the current-time integral (Fig. 6A). The correlation coefficient is 0.84 in the relatively light-adapted state (n=10) and 0.88 in the relatively dark-adapted state (n=15). The regression coefficients are not significantly different ($P>0.5$) for the two states of adaptation. Test flashes of the lowest light intensity necessary to evoke detectable arsenazo signals in both states of light adaptation caused receptor currents with time integrals of about 3–5 nC. Stimulus intensities leading to almost saturated arsenazo signals in the relatively dark-adapted photoreceptor caused receptor currents with time integrals of 100–500 nC. In addition, the time-to-peak of the arsenazo signal is correlated with the current-time integral (Fig. 6B). The correlation coefficient is 0.79 in the relatively

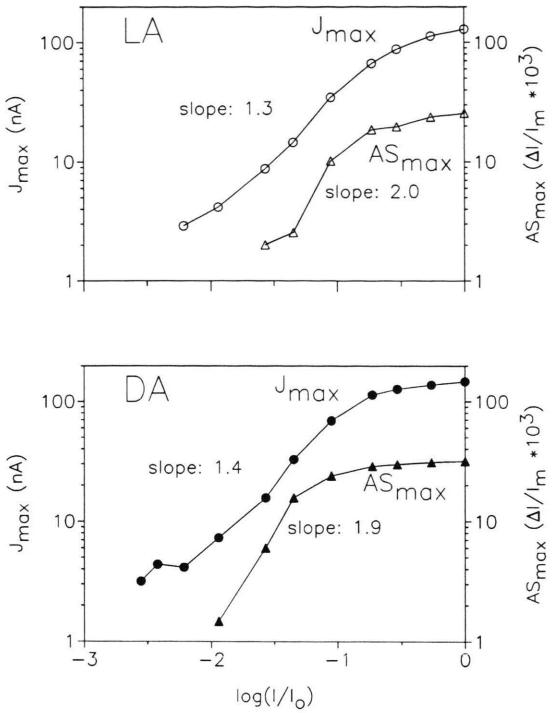

Fig. 4. Log-log plots of receptor current amplitude (J_{max}, open circles) and arsenazo signal (AS_{max}, open triangles) versus the stimulus intensity (I_o= 5×10^{14} photons/cm^2). These curves were shown for the relatively light-adapted state (LA) with open symbols and the relatively dark-adapted state (DA) with filled symbols. Slopes were calculated between the two points with maximum steepness (same experiment as in Figure 3).

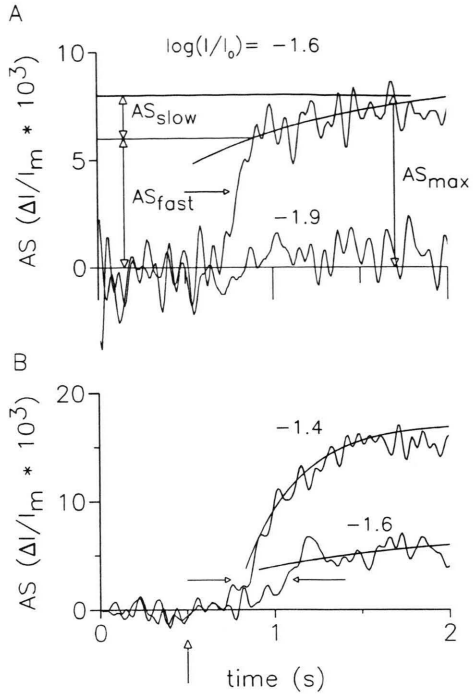

Fig. 5. Arsenazo signal evoked by flashes of different light intensities from the supralinear range are shown for two cells (in B: same experiment as in Figs. 3,4). The test flash is indicated by a vertical arrow. The stimulus intensity is presented in $\log(I/I_o)$ with $I_o=5\times10^{14}$ photons/cm^2. The fit curve of the slow rising phase is plotted. The fast phase in the rise of the signals is indicated by horizontal arrows. A, shows the rise of arsenazo signals leading to supralinearity where only the larger arsenazo signal consists of two rising phases. The fast phase mainly contributes to the total amplitude of the larger arsenazo signal. B, shows arsenazo signals from a different cell. Supralinearity in this case is mainly due to the increase in the slow phase of the signal rise.

light-adapted state (n=10) and 0.72 in the relatively dark-adapted state (n=15). Regression lines were calculated for the data in each state of adaptation. Again, these regression coefficients are not significantly different (P>0.5).

Controls

We investigated the influence of the photometric beam without injection of arsenazo on the maximal slope of receptor current-versus-stimulus intensity curves measured in strontium saline with 0.25 mM calcium. The illuminating photometric beam did not visibly influence the slope of these curves (Table IC,D). In addition, we determined the influence of intracellular arsenazo on these curves. The injection of arsenazo III without measuring beam did not influence the maximal slopes (Table IB,D). The influence of extracellularly applied Sr^{2+} on the receptor current-versus-stimulus intensity characteristics was tested in cells which were first superfused with physiological saline while stimulated as described above and subsequently with strontium saline with 0.25 mM calcium for 45 min. These experiments were carried out without the injection of arsenazo III, and the photometric light beam was turned off. Log-log plots of J_{max} versus the stimulus intensity show supralinear regions in both states of light adaptation (Fig. 7). In physiological saline the steepest slope of the curves was on average 1.8 (2 cells; Table II) in both states of adaptation. In strontium saline the steepest slope of the curves was on average 1.5 for the light-adapted photoreceptor

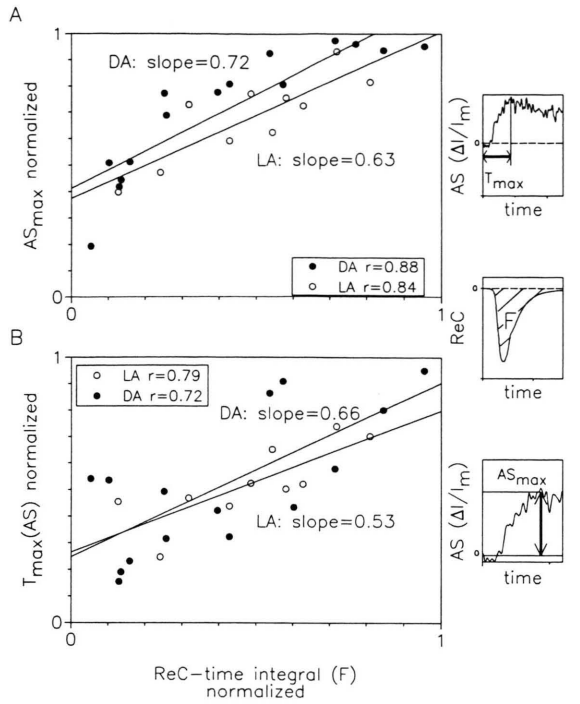

Fig. 6. Correlation plots of arsenazo signal peak amplitude or time-to-peak versus current-time integral from 4 response-versus-stimulus intensity measurements. Light responses were evoked in a relatively dark-adapted state (DA, closed circles) and a relatively light-adaptated state (LA, open circles) when the nerve was superfused with strontium saline with 0.25 mM calcium. Regression lines are shown for each adaptation state. Parameters (illustrated at the right side) of each experiment were normalized to the largest value found in each state of adaptation. This largest value is 354 ± 211 nC for the relatively dark-adapted state and 355 ± 175 nC for the relatively light-adapted state (as means ± SD). A, normalized AS_{max} is plotted versus the normalized current-time integral (F). Largest value of AS_{max}: 0.028 ± 0.009 $\Delta I/I_m$ (DA; Pearsons correlation coefficient (r): r=0.88; n=15) and 0.03 ± 0.008 $\Delta I/I_m$ (LA; r=0.84; n=10). The slopes of the regression lines are 0.72 (DA) and 0.63 (LA), and they are not significantly different (P > 0.5). B, normalized time-to-peak of the arsenazo signal (T_{max}(AS)) is plotted versus the normalized current-time integral (F). Largest value of T_{max}(AS): 6.4 ± 3.7 s (DA) and 5.9 ± 3.7 s (LA). Pearsons correlation coefficient (r) is 0.72 (n=15; DA) and r=0.79 (n=10; LA). The slopes of the regression lines are 0.66 (DA) and 0.53 (LA) and they are not significantly different (P > 0.5).

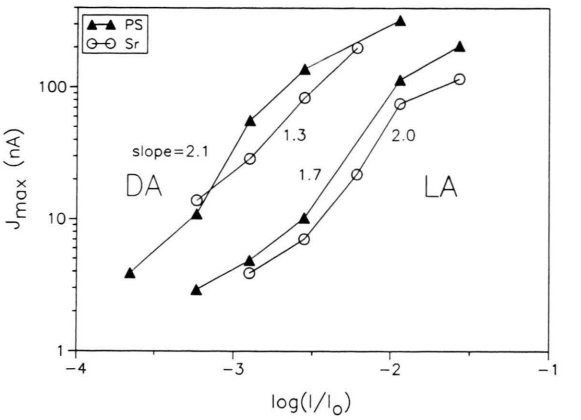

Fig. 7. Log-log plot of current peak amplitude (J_{max}) plotted versus the normalized stimulus intensity (I_o= 5×10^{14} photons/cm^2). Data measured in PS (triangles) and 45 minutes after exchange of the superfusate to strontium saline with 0.25 mM calcium (circles). Responses to flashes from the dark adapted photoreceptor (DA) and 18 s after a light adapting stimulus (LA) are shown. Slopes were calculated between the two points with maximum steepness.

Table II. Average values for the maximum slopes of receptor current-versus-stimulus intensity curves in two states of light adaptation (as means ± SD; n=2 cells). Measurement without arsenazo and without photometric beam. Slopes are calculated between the two points with maximal steepness in a light-adapted state (LA) and a dark-adapted state (DA) from data recorded in physiological saline and 45 min after the nerves were superfused with strontium saline with 0.25 mM calcium.

	Maximal slopes			
	Physiological saline		Strontium saline with 0.25 mM Ca	
	LA	DA	LA	DA
J_{max}	1.8 ± 0.1	1.8 ± 0.4	1.5 ± 0.4	1.3 ± 0.003
Current-time integral	1.3 ± 0.2	1.7 ± 0.3	1.6 ± 0.4	1.0 ± 0.2

and 1.3 for the dark-adapted cell. Both in physiological and strontium saline the curves of receptor currents and arsenazo signals in the light-adapted state were shifted by about 0.5 log units towards higher stimulus intensities compared to the curves for the dark-adapted state.

Discussion

The rising phase of the arsenazo signal consists of two phases with different kinetics. The amplitude of the slow rising phase contributes to 20–70% to the overall amplitude of the arsenazo sig-

nal. Replacement of external calcium in the superfusate by strontium led to an increase in the amplitude of the slow rising phase. The amplitude of the fast rising phase increased only transiently and was no longer detectable after about 2 h in strontium saline without calcium. However, the fast rising phase remained detectable for even more than 2 hours in strontium saline if 0.25 mM calcium was added to the superfusate. In strontium saline with 0.25 mM Ca the kinetics of the slow rising phase depended on the membrane voltage and was slowed down with positive membrane voltages. The fast rising phase, however, was not detectably voltage-dependent. In the same saline, supralinear sections could be observed in log-log plots of response-versus-stimulus intensity curves. This supralinearity was found for the receptor current peak amplitude, the current-time integral and the arsenazo signal amplitude, in both states of light adaptation. Supralinearity occurred at least within 0.3 log units of identical stimulus intensities in the arsenazo signal and the receptor current. Supralinearity was observed both for the amplitude of the fast rising phase of the arsenazo signal as well as for the amplitude of the slow rising phase (Fig. 5). The total amplitude of the arsenazo signal as well as the time-to-peak correlated with the receptor current-time integral. Regression coefficients from these correlation plots were not significantly different in both adaptational states. Light adaptation by a conditioning stimulus caused a parallel shift of the curves towards higher intensities. The extent of the shift was similar when the nerve was superfused with physiological saline or strontium saline. The slope of the receptor current-versus-intensity curves measured in physiological saline and in strontium saline was less steep in our study compared to previous results (Stieve *et al.,* 1986; Stieve and Schlösser, 1989). This may be partially due to the incomplete voltage clamp (see Materials and Methods).

Stimulus intensity and photoresponse

Supralinearity in the arsenazo peak amplitude-versus-stimulus intensity curves might indicate a cooperative step within the transduction cascade leading to calcium release from internal stores. On the other hand, the observed supralinearity might indicate that the cellular buffers for calcium or strontium are saturated with a certain stimulus intensity. A further increase in the stimulus intensity would then lead to an increase in cytosolic calcium or strontium which overwhelms the cellular buffer. As a consequence the arsenazo signal peak amplitude would increase disproportionate to the stimulus intensity. This possibility cannot be fully excluded, but one important finding is, that we observed supralinearity within a similar range of stimulus intensity for the light-adapted cell compared to the relatively dark-adapted cell. Since in the light-adapted cell the cytosolic calcium or strontium concentration is higher than in the relatively dark-adapted cell we would expect that supralinearity due to saturation of cellular calcium buffers can be observed within a range of lower stimulus intensities in the light-adapted cell compared to the relatively dark adapted cell. In contrast, in some cases supralinearity was observed for a lower range of stimulus intensity in the relatively dark-adapted cell compared to the light-adapted cell (Fig. 4). Assuming, that supralinearity in response-versus-stimulus intensity curves for the arsenazo signal indeed reflects a cooperative process within the transduction cascade our data allow to analyse this supralinearity in more detail. The rise of the arsenazo signal consists of two phases, an initial fast phase indicating calcium release from internal stores and a slow further increase due to calcium influx probably through light-activated channels (Stommel and Stieve, 1995; Stommel *et al.,* 1996). The interpretation above proposed that only one cooperative step is involved in the cascade leading to calcium increase might be too simple because in this study we find that the amplitudes of both phases increase supralinearly with the stimulus intensity. Supralinearity of the amplitude of the fast rising phase of the arsenazo signal, which is not detectably voltage dependent, might be due to cooperativity in the calcium release mechanism. IP_3-sensitive Ca^{2+} release is cooperative in rat basophilic leucemia cells (Meyer, Wensel and Stryer, 1990). These authors suggest that 4 IP_3 molecules are necessary to activate the tetrameric IP_3-receptor. Accordingly we propose the interpretation that the supralinear increase in the amplitude of the fast rising phase of the arsenazo signal is due to the fact that more than one IP_3 molecule is necessary to activate an IP_3-receptor in the membrane of internal calcium

stores. If calcium participates in the generation of the electrical light response (Payne *et al.,* 1986b), supralinearity of the receptor current could be due to cooperativity in the calcium release mechanism.

The amplitude of the slow rising phase of the arsenazo signal showed supralinearity, too. The amplitude of the arsenazo signal measured in strontium saline is mainly determined by the size of this slow rising phase. If the slow rising phase indicates calcium influx, a correlation of the arsenazo signal with the receptor current-time integral would be expected. This calcium influx hypothesis agrees with our finding that the peak amplitude and the time-to-peak of the arsenazo signal correlate with the current-time integral. This correlation was found for the relatively light- and dark-adapted cell indicating that the arsenazo signal indeed depends on the receptor current independent of the adaptional state of the cell. Light-stimulated influx of calcium was already shown for barnacle, honey bee and *Drosophila* photoreceptors (Ziegler and Walz, 1989; Hardie, 1991; Werner, Suss-Toby, Rom and Minke, 1992; for review see Minke and Selinger, 1996). In *Limulus* ventral photoreceptors one type of light-activated receptor current component is blocked by *l-cis*-diltiazem similarly to the cGMP-gated current of rods (Nagy, 1994). Since rod channels are calcium permeable, the same might apply to the *l-cis*-diltiazem-affected channels in *Limulus.* This type of channels presumably generates the C1 component of the receptor current in *Limulus* (Nagy, 1994). The receptor current attributed to the C1 component increases proportionally to the stimulus intensity (Deckert *et al.,* 1992). Therefore it seems unlikely that this calcium-carrying C1 current causes the supralinearity of the slow rising phase of the arsenazo signal. A receptor current attributed to the C2 component showed supralinearity in response-versus-stimulus intensity curves (Deckert *et al.,* 1992; Contzen and Nagy, 1996). Since the C2 component is the only component were supralinearity of the receptor current was observed we assume that C2 is partially carried by calcium, and causes supralinearity of the slow rising phase in arsenazo signals. The receptor current component C2 is activated via the IP_3-dependent calcium release pathway (Contzen and Nagy, 1995). Thus, an IP_3 activated calcium release from intracellular stores is required for the activation of the C2 current component. If we assume that this C2 current component carries calcium besides other cations, two different sources of calcium which are activated within the PLC pathway are monitored by the two different phases of the arsenazo signal. Supralinearity of both rising phases in the arsenazo signal might therefore be due only to the cooperative calcium release mechanism whithin the PLC pathway.

Effects of strontium

The replacement of calcium by strontium in the superfusate enabled us to evoke detectable arsenazo responses even with relatively low stimulus intensities. The minimum light intensity of the test flash necessary to evoke a detectable arsenazo signal caused a receptor current which carried about 3–5 nC net charge into the cell. The most active channel type found in the plasma membrane of *Limulus* ventral photoreceptors has a conductance of about 10 pS and a mean open time of about 1.4 ms (Nagy and Stieve, 1990). At -50 mV membrane potential about 10^6 of these channels would have been transiently activated to cause a receptor current of 3–5 nC. Additional channel types with conductances of about 6 and 30 pS (Nagy and Stieve, 1990) or 40 pS (Bacigalupo *et al.,* 1986) have been reported. With regard to these variations and assuming a range for the membrane voltage of -80 mV to -40 mV the number of transiently opened channels might vary between 7×10^5 and 2×10^7.

The receptor current and the arsenazo signal are enlarged in strontium saline, which was explained by a weaker desensitizing action of Sr^{2+} compared to Ca^{2+} (Stieve and Benner, 1992). However, injections of Ca^{2+} and Sr^{2+} into *Limulus* ventral photoreceptors caused both excitation and desensitation of the photoreceptors (Payne *et al.,* 1986a). We found that a conditioning stimulus light-adapted the cell and shifted receptor current-versus-stimulus intensity curves to higher intensities in strontium saline to the same extent as in physiological saline.

More than one mechanism of invertebrate phototransduction is calcium-dependent. Cytosolic calcium inhibits calcium release from internal stores in *Limulus* ventral photoreceptor (Payne, Flores and Fein, 1990). In *Drosophila* photorecep-

tors one type of the light-sensitive channels is directly inactivated by calcium (Hardie and Minke, 1994). One possible explanation for the prolongation of the photoresponse in strontium saline could be that a direct inactivation of the ion channels in the plasma membrane by calcium is not or only weakly adopted by strontium. Then it has to be assumed that strontium mimics those desensitizing functions of calcium which lead to sensitivity shift caused by conditioning flashes to about the same extent as calcium.

The contribution of strontium and calcium to the receptor current

In *Limulus* ventral photoreceptors, the cytosolic free calcium concentration within the rhabdomeric lobe increases to about 60–70 μM due to strong light stimuli (Ukhanov *et al.*, 1995). After exchange of the superfusate from physiological saline to strontium saline the arsenazo signal amplitude caused by strong light stimuli increases 3–5 fold (see also Stieve *et al.* 1994). Therefore we assume that the cytosolic free strontium concentration increases to 180–350 μM. Different values of K_D might lead to an error in this calculation. Because the cytosolic free concentration of calcium or strontium is at least 10 times larger than the value of the K_D for strontium or calcium, this error is maximally 5% for the estimated strontium concentration and will not be considered further. In parallel to these large arsenazo signals we find receptor-current-time integrals between 100 and 500 nC. The volume of a large photoreceptor cell is up to 400 pl (Corson and Fein, 1983). If we assume that in strontium saline the arsenazo signal is mainly due to influx of strontium through light-activated channels and that light flashes which saturate the arsenazo response cause an increase in strontium to 180–350 μM and receptor current-time integrals of 100–500 nC, the contribution of strontium to the receptor current-time integral is about 3–30% in cells with a volume of 400 pl. For this calculation the estimated peak strontium concentration (180–350 μM) is divided by the calculated strontium concentration, assuming that 100% of the ReC (100–500 nC) would be strontium ions. The mathematical procedure is: contribution of strontium ions to the receptor current (%) = $[Sr^{2+}]_i$•2•Faraday constant•cell volume/current-time integral. Data from Stieve and Benner (1992; their Fig. 6b) measured in physiological saline show that the arsenazo signal amplitude might not be saturated even with strong stimuli. The receptor current-time integral corresponding with the largest arsenazo signal is about 400 nC. Assuming 400 nC receptor current-time integral and 60–70 μM calcium in a cell of 400 pl volume, the contribution of calcium to the receptor current-time integral is about 1–1.5%. These calculations were done without regard to cellular buffers for calcium and strontium or transport mechanisms which reduce cytosolic divalent cations. The absolute values for calcium or strontium influx might be influenced by these mechanisms. Nevertheless our results allow the rough estimation that strontium might contribute about 10 times more to the receptor current than calcium does.

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