# Effects of Calcium and Cyclopiazonic Acid on the Photoresponse in the *Limulus* Ventral Photoreceptor

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1. Single-photon responses (bumps) and small macroscopic photocurrents were studied in ventral photoreceptors of the horseshoe crab *Limulus*. Lowering the calcium concentration in the bath from 10 mM to 250 μM led to increased bump size. Adaptation of the cells by a moderately bright conditioning flash was not impaired.

2. Pressure-injection of 1.2 mM EGTA into the dark-adapted cells resulted in reduced bump size. EGTA weakened the effect of the conditioning light flash although it did not completely abolish light adaptation.

3. The microsomal calcium-ATPase inhibitor cyclopiazonic acid strongly desensitized the cells, and bumps were suppressed below detection. When the bathing saline contained 10 mM calcium, macroscopic photoresponses after extracellular application of the agent had amplitudes smaller than under control conditions but normal response kinetics: The response to a light step still consisted of a fast transient photocurrent and a much smaller plateau. However, when applied in calcium-free bathing saline, cyclopiazonic acid additionally influenced the waveform of the photoresponse. The clear distinction between transient and plateau was no longer possible, and the photocurrent appeared "square".

4. Our results support the idea that a transient elevation of the cytosolic calcium concentration is obligatory for light adaptation in the ventral photoreceptor. It is also obligatory for the generation of the so-called $C_2$ component of the photocurrent which is represented by "standard" bumps and the fast transient phase of a prolonged response. However, a rise in cytosolic calcium appears not necessary for the initiation of a slow electrical photoresponse.

## Introduction

Illumination of invertebrate microvillar photoreceptors evokes a depolarization, the receptor potential, which is based on an inward flux of positively charged ions (reviewed in Minke and Selinger, 1991; Dorlöchter and Stieve, 1997). In the ventral photoreceptor of the horseshoe crab *Limulus polyphemus* the absorption of a single photon leads to a small transient receptor current or depolarization called "quantal bump" (Yeandle, 1958). Bright illumination evokes a macroscopic photoresponse that consists of many temporarily and spatially fused bumps (Dogde, Knight and Toyoda, 1968; Brown and Coles, 1979; Stieve, 1986).

The electrical photoresponse is accompanied by a rise in the concentration of free calcium ions in the cytoplasm (Brown, Brown and Pinto, 1977; Maaz and Stieve, 1980; Levy and Fein, 1985). In the ventral photoreceptor the endoplasmic reticulum forms an extensive network in which calcium is stored and from which it is released after illumination (Payne, Walz, Levy and Fein, 1988; Feng, Carson, Morgan, Walz and Fein, 1994). In addition to release from internal stores calcium influx from the extracellular space contributes to the transient augmentation of the cytosolic free calcium concentration (Stommel, Dorlöchter, Rüsing and Stieve, 1996).

Two major roles in the phototransduction process have been attributed to calcium ions. It is widely accepted that they control the sensitivity of the photoreceptor cell. Illumination not only excites but at the same time desensitizes (adapts) the photoreceptor (Lisman and Brown, 1972, 1975; Fein and Lisman, 1975; Levy and Fein, 1985). More controversially discussed is the role of calcium ions in the excitation process. Injection of

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calcium into ventral photoreceptors increased the light response in calcium-deprived cells, or elicited inward currents similar to those caused by light so that an obligatory role for calcium in excitation was proposed (Bolsover and Brown, 1985; Payne, Corson and Fein, 1986b; Shin, Richard and Lisman, 1993). On the other hand, application of calcium buffer or agents blocking calcium release did not completely inhibit the photocurrent indicating calcium-independent initiation of a light response (Frank and Fein, 1991; Contzen, Richter and Nagy, 1995).

The contribution of calcium to the generation and light adaptation of single-photon responses and small macroscopic currents, respectively, was analyzed. Single-photon responses were recorded under dark- and light-adapted conditions in varied calcium concentrations of the bath solution and after EGTA microinjection. We also manipulated the contents of the internal calcium stores by using intense illumination in order to deplete them. An inhibitor of the microsomal calcium-ATPase was employed for the same purpose. We had already shown that one type of single-photon responses is not much affected by changes in intra- or extracellular calcium (Dorlöchter, Yuan, Hennig and Stieve, 1996). Here we demonstrate that calcium withdrawal does not abolish the generation of macroscopic currents although the cells are strongly desensitized.

Short parts of this study have been published earlier (Yuan, Hennig and Stieve, 1995; Dorlöchter, 1997; Dorlöchter and Stieve, 1997).

## Methods

If not stated otherwise, all experiments were performed the following way: Ventral nerves from male *Limulus polyphemus* (Marine Biological Laboratory, Woods Hole, MA, USA) were dissected and stored in organ culture medium at 4 °C for 18–42 hours (Kass and Renninger, 1988). Before the experiments nerves were treated with collagenase or pronase (2 mg/ml physiological saline) for 4–10 min and mounted in a test chamber which was continuously perfused with test solution.

Photoreceptors were impaled with glass micropipettes filled with 0.5 M KCl solution (tip resistance 4–15 megohms). Current measurements were performed using a double- or a single-electrode voltage clamp device (Biologic, France). Throughout the experiment membrane voltage was clamped at the resting (dark) potential of the cell, which was usually −40 to −80 mV.

### *Bump recordings*

Photoreceptors were continuously illuminated with dim light ($7x10^6$ to $4x10^7$ photons/cm$^2$×s) from a halogen lamp passed through a wide band interference filter (515 nm maximum). At the beginning of the experiment the cells were light-adapted and did not respond to the dim light. After around 30 min small bumps appeared and their amplitudes increased with growing dark adaptation. When the size of the bumps had become stable, light intensity was adjusted so that on average 0.5–1.0 bumps were recorded per second. From then on every 16 s traces of 10 s duration were digitized (at 1 kHz) and stored on computer disk. This cycle was repeated 60–100 times for each phase of the experiment. To cause light adaptation, a moderately bright conditioning stimulus from a flash lamp was administered 2.5 s prior to the 10 s recording. The intensity of this flash was adjusted in the first phase of the experiment for each cell so as to reduce bump size to about 50%. In some experiments with lowered bath calcium concentration, 3–6 very bright flash stimuli ($6.4x10^{14}$ photons/cm$^2$) were delivered 60 min prior to the next experimental phase in order to exhaust the intracellular calcium stores (Maaz and Stieve, 1980; Bolsover and Brown, 1985).

Experiments were carried out in physiological saline (10 mM $Ca^{2+}$, 481 mM $Na^+$, 10 mM $K^+$, 55 mM $Mg^{2+}$, 10 mM HEPES, pH 7.5) or in a solution containing reduced amounts of calcium ("low Ca": 250 μM $Ca^{2+}$, 481 mM $Na^+$, 10 mM $K^+$, 65 mM $Mg^{2+}$, 10 mM HEPES, pH 7.5). Test solutions were exchanged under dim continuous light. About 30 min after switching solutions bump recordings were started again. The calcium buffer EGTA was pressure-injected into the cells at a concentration of 1.2 mM in the electrode tip. With a cell volume of several hundred picoliters (Corson and Fein, 1983) and 10–50 pl liquid injected, the final concentration in the cell is estimated as about 0.1 to 0.01 mM. Bump recordings were started about 1 min after the injections. Temperature was kept constant at 15 °C.

*Experiments with cyclopiazonic acid (CPA)*

Photoreceptors were illuminated for 4–6 s at varying intensities. To allow dark adaptation between the recordings, the cycle was repeated only every 3 min.

In the first part of these experiments, cells were superfused with physiological saline (10 mM calcium, see above) or with saline containing no added calcium and 1 mM EGTA ("calcium-free") for at least 30 min before the measurements started. At the end of the recordings, superfusion was stopped, and 0.2 ml of a 1 mM solution of CPA (Calbiochem-Novabiochem, Bad Soden, Germany) in 10% dimethyl sulfoxide (DMSO) were added to the bath resulting in a final concentration of about 100 μM CPA in 1% DMSO in physiological or calcium-free saline, respectively. For controls, 0.2 ml of 10% DMSO were added to the bath. In the unclamped photoreceptor this led to a transient depolarization. After about 5–10 min the dark membrane potential recovered. In the clamped cell, no obvious effect was observed on either bump number, size, or shape. Experiments were done at room temperature.

*Data analysis*

Signals were sampled at 1 kHz and stored on disk. They were digitally filtered with a 50 Hz notch filter and a 70 or 90 Hz low pass filter and analyzed with computer software, as described earlier (Reuß and Stieve, 1993). For statistical comparison of means paired t-tests were applied.

## Results

*Bumps in dark and light adaptation, in normal and reduced calcium concentrations, and after EGTA injection*

Figure 1A shows the illumination and recording protocol used in the experiments: In the dark-adapted photoreceptor bumps were induced by continuous illumination with dim light. To cause light adaptation, a conditioning flash of moderate brightness was applied 2.5 s prior to the recording period. Bumps were studied in physiological saline (10 mM $Ca^{2+}$) and in a solution containing only 250 μM $Ca^{2+}$ ("low Ca"). Recordings were also done in low Ca solution after illuminating the cells

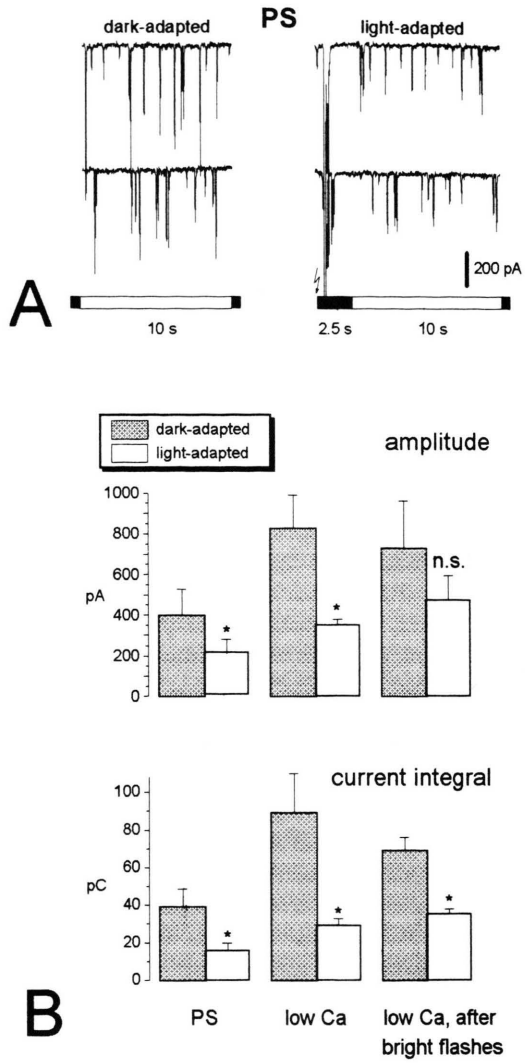


Fig. 1A. Recordings showing single-photon responses (bumps) in a ventral photoreceptor bathed in physiological saline (*PS*). Bumps were evoked by dim continuous illumination of the dark-adapted photoreceptor (*left panel*) or in a slightly light-adapted state after a conditioning flash of moderate brightness (*right panel*). Periods of 10 s duration were recorded as indicated by the open part of the bar below the traces. The photoreceptor was clamped at -82 mV. – B. Bump size in dark- and light-adapted ventral photoreceptors bathed in physiological saline (*PS*), then in saline containing only 250 μM calcium (*low Ca*). In a third phase of the experiment, cells in low calcium saline were illuminated with several bright flashes 60 min prior to the start of the recordings (*low Ca, after bright flashes*). Data are expressed as means ± S.E.M, $n = 4$ cells. In each experimental phase of a photoreceptor about 100–500 bumps were evaluated and data averaged. * significantly different from the value in dark adaptation, $p < 0.05$; n.s. not significant (paired t-test).

with 3–6 very bright flashes and after the injection of 1.2 mM EGTA.

Data from four photoreceptors which were stable enough to perform all three phases of the experiment consecutively, is summarized in Figure 1B. Our results confirm those from earlier studies (reviewed in Stieve, 1986) and are described only briefly. In physiological saline, the conditioning flash led to light adaptation and the average bump amplitude and current integral were smaller by 48% and 59%, respectively. With reduced bath calcium concentration, bumps recorded from the dark-adapted photoreceptors were about twice as large as in physiological saline. Under these conditions the ability of the cells to adapt was even better than with normal external calcium concentration. After the conditioning flash, in low calcium saline bump amplitudes and current integrals were reduced to about 40% and 30% of the respective average values in dark adaptation.

Repetitive illumination of the photoreceptor in addition to reduced external calcium concentration prevent the light-induced increase in cytosolic calcium (Maaz and Stieve, 1980; Bolsover and Brown, 1985). While the cells were still bathed in low Ca solution, in the third experimental phase we therefore applied bright flashes to further deplete the cells of calcium by exhausting the intracellular calcium stores. After several bright flashes, photoreceptors did not adapt as well as before the flashes. The conditioning stimulus led to significant diminution of only the average current integral, while the reduction in the average bump amplitude was not significant (Fig. 1B).

In order to buffer the transient light-induced increase in free cytosolic calcium ions, 1.2 mM

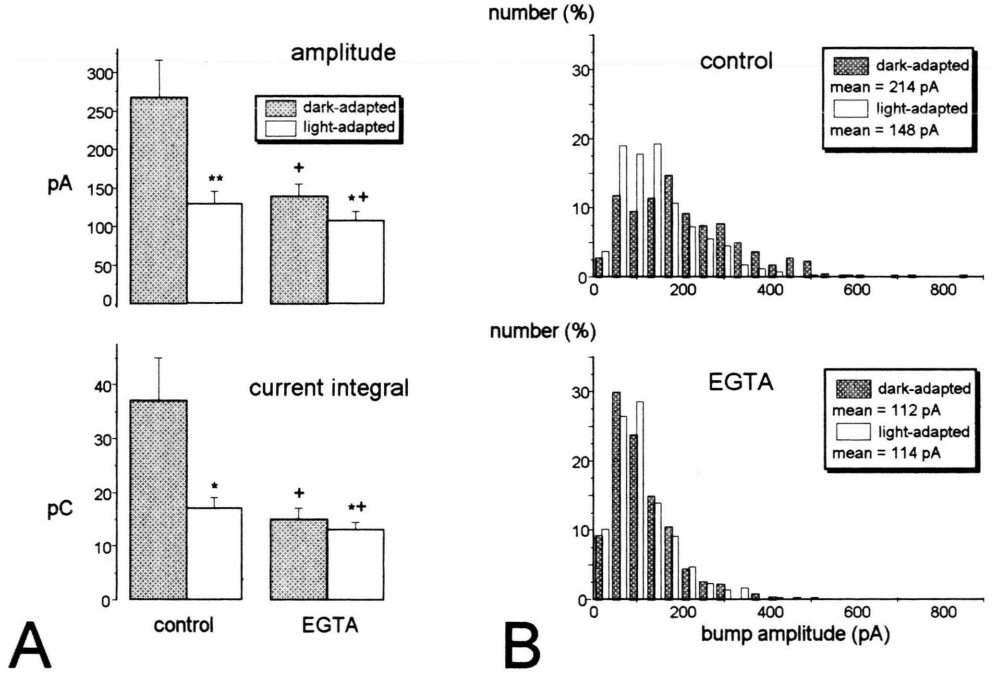


Fig. 2A. Bump size in dark- and light-adapted ventral photoreceptors bathed in physiological saline before (*control*) and after (*EGTA*) the microinjection of 1.2 mM EGTA solution. The amplitudes and current integrals are expressed as means ± S.E.M, $n = 8$ cells. In each photoreceptor about 200–400 bumps were evaluated before and after EGTA injection and data averaged. *,** significantly different from the value in dark adaptation, $p < 0.05$ and $p < 0.01$, respectively (paired t-test); + significantly different from the respective control value (paired t-test). – B. Histograms showing the distribution of bump amplitudes from a ventral photoreceptor bathed in physiological saline before (*control*) and after (*EGTA*) the microinjection of 1.2 mM EGTA solution. Light adaptation under control conditions shifts bump amplitudes towards smaller values without greatly influencing the shape of the distribution. After the injection of EGTA, bumps are smaller in the dark-adapted state. There is no additional shift towards smaller bump amplitudes in the light-adapted state.

EGTA was injected into photoreceptors bathed in physiological saline (8 cells). Intracellular EGTA led to decreased average bump size. In the dark-adapted state, bump amplitudes were reduced to about half their size prior to the EGTA injection (Fig. 2A). The effect of EGTA injection was dose-dependent and was more pronounced with additional successive injections of 1.2 mM EGTA. Similarly, already the first injection of 5–10 mM EGTA completely abolished bumps (not shown).

Impairment of light adaptation after injections of calcium-buffering substances had been demonstrated for macroscopic photocurrents (Lisman and Brown, 1975; Payne *et al.,* 1986a). Similarly, on the bump level, EGTA-injected cells partially lost their capability to adapt. After the conditioning stimulus, bump amplitudes and current integrals were further reduced, however to a smaller extent than before EGTA injection. While before the EGTA injection bump amplitudes were smaller in the light-adapted state by 52% on average, after EGTA injection they decreased by only 22%. This smaller light-adapting effect of the conditioning flash was found in each cell tested. In two of the 8 cells, bumps were of the same size or even slightly larger after the conditioning flash. One might argue that this finding is an artifact because with EGTA bumps were already smaller in the dark-adapted state. If the whole population of already small bumps were shifted towards even smaller values, one could expect that a high proportion of signals may be lost in the noise. It appears possible that under these conditions only the largest bumps were detectable, which may be misinterpreted as a weaker effect of the conditioning flash. However, after EGTA injection, the bump rate was on average 0.6 $s^{-1}$ both in the dark- and light-adapted state. We also checked the frequency distributions of bump amplitudes in each photoreceptor. Figure 2B shows the results from a photoreceptor before and after the injection of EGTA, in the dark-adapted state and after the conditioning stimulus. The smallest bin in both frequency distributions after EGTA injection contains 10% of the data or less. Thus, from the shapes of the distributions it appears unlikely that more than 5–10% of the bumps were hidden in the noise, neither before nor after the conditioning stimulus. In this particular cell, before EGTA injection the average bump amplitude was reduced to 70% of its dark-adapted value by the conditioning stimulus, while after EGTA injection it remained virtually unchanged (102%).

### *Effects of the calcium-ATPase inhibitor cyclopiazonic acid*

Cyclopiazonic acid (CPA) induces calcium leakage from internal stores in various vertebrate and invertebrate tissues and inhibits the calcium-ATPase in the endoplasmic reticulum. The agent thus prevents refill of the stores after calcium release and leads to their depletion (Mason, Garcia-Rodriguez and Grinstein, 1991; Demaurex, Lew and Krause, 1992; Walz, Baumann, Zimmermann and Ciriacy-Wantrup, 1995; Ukhanov and Payne, 1995). We recorded photoresponses in physiological saline before and after the addition of CPA to the bath solution (6 cells). CPA led to rapid dose-dependent desensitization of the cells. At a final concentration of about 50 μM CPA in the bath solution, illuminating the photoreceptor with dim light immediately after the addition of the agent evoked bumps. However, their amplitude gradually decreased and they were no longer detectable after 7 illumination cycles (21 min). At a final CPA concentration of 100 μM, no bumps were visible already in the first recording cycle, about 3 min after the CPA addition. With increased stimulus intensity the cells responded with small, more or less "square" macroscopic inward currents (Fig. 3) while at still higher light intensities the photoresponse started with a fast transient followed by a much lower plateau. The kinetics of these responses appeared similar to that in physiological saline. However, the cells were less sensitive by about 2–3 log units.

The effect of CPA in calcium-free saline on photoreceptors (4 cells) was quite different. Figure 4 shows results from a photoreceptor bathed in physiological saline, then in calcium-free saline for about 60 min. Finally, CPA was added and recordings restarted after about 30 min incubation in darkness. In calcium-free saline bumps and macroscopic photocurrents at a given light intensity were larger than in PS. In addition, latencies were prolonged and the kinetics of the transient part of the response slightly slower. The ratio of the transient to the plateau phase, however, was not changed (Fig. 4A). After the addition of 100 μM CPA to the

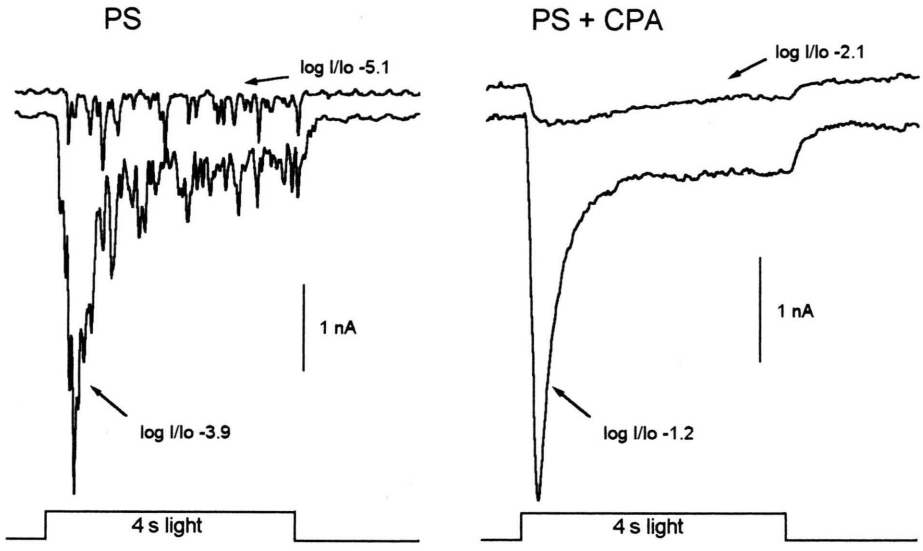


Fig. 3. Recordings from a ventral photoreceptor bathed in physiological saline before (*PS*) and after (*PS + CPA*) the addition of cyclopiazonic acid to the bath. The final concentration of CPA was 100 μM. In physiological saline single bumps were evoked with dim illumination. They superposed to build a small macroscopic current with a transient and plateau phase at higher light intensity. After CPA treatment the cell was strongly desensitized. No bumps were detectable and the light intensity necessary to evoke photocurrents of a similar maximum amplitude was about 2–3 log units higher. The general waveform of the photoresponse and the ratio of the transient and plateau phases was not much influenced by CPA. The photoreceptor was clamped at −56 mV.

calcium-free saline, the cell was strongly desensitized with no bumps detectable. The macroscopic currents in this cell remained more or less "square" even at relatively high light intensities, and it was not possible to distinguish between a fast transient and a plateau phase (Fig. 4B). In Fig. 4C response *versus* intensity curves are plotted for this photoreceptor, showing the increased sensitivity of the cell in calcium-free saline as compared to normal calcium conditions, and the decreased sensitivity after CPA application.

## Discussion

### *Effect of calcium on response generation and adaptation*

Single-photon responses and small macroscopic currents in ventral photoreceptors were measured under various conditions of adaptation and calcium withdrawal. Under low extracellular calcium conditions, the photoreceptor is more sensitive to light, and macroscopic and single-photon responses are larger. It was therefore proposed that calcium ions are inhibitory on the phototransduction cascade (Millecchia and Mauro, 1969; Lisman and Brown, 1975; Bolsover and Brown, 1985; Levy and Fein, 1985; Payne *et al.*, 1988; Stieve, Reuß, Hennig and Klomfaß, 1991; present study). Using fura-2 Ukhanov, Flores, Hsiao, Mohapatra, Pitts and Payne (1995) found that after about 10 min in calcium-free saline the resting $Ca_i$ in the dark-adapted cell was reduced from an average of 0.46 μM to 0.2 μM. In our experiments the saline still contained 250 μM calcium so that the reduction in $Ca_i$ was probably less pronounced. However, it was sufficient to weaken the inhibitory effect of calcium, which resulted in increased bump size.

Intracellular application of 1.2 mM EGTA affected the photoreceptor contrarily to extracellular calcium reduction. The amplitudes of single-photon responses decreased by about 50%, and bumps were completely abolished by injection of higher EGTA concentrations. EGTA and other calcium chelators act by buffering the light-induced transient increase in cytosolic calcium (Bolsover and Brown, 1985; Levy and Fein, 1985; Payne *et al.* 1986a,b). Thus, calcium ions have a

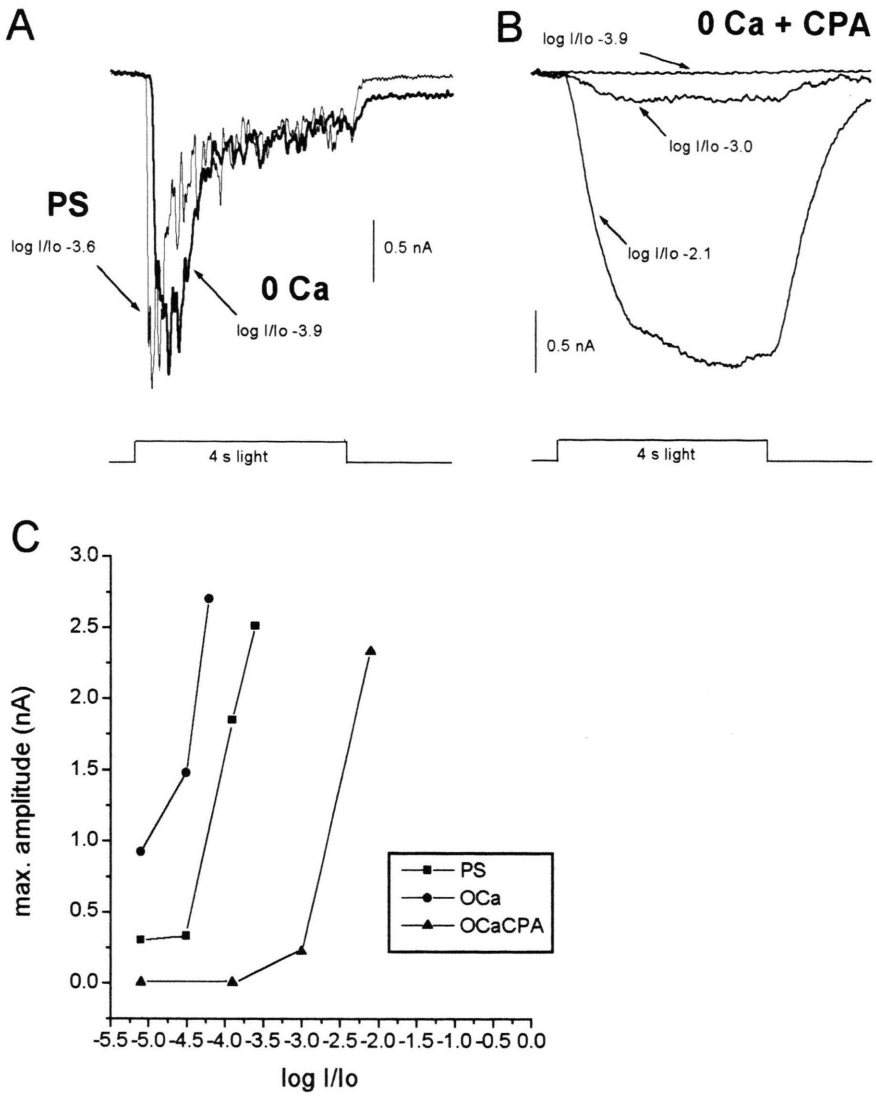


Fig. 4. Effects of calcium and cyclopiazonic acid on photocurrents in a ventral photoreceptor. – A. Recordings from the photoreceptor bathed in physiological saline (*PS*) and in saline containing no added calcium and 1 mM EGTA (*0 Ca*). After 30 min superfusion with calcium-free saline in the dark, the cell was more sensitive to light. The light intensity necessary to evoke a response of similar amplitude as in PS was about 0.3 log units lower than in normal calcium. – B. Traces from the same cell after the addition of cyclopiazonic acid to the bath (*0 Ca + CPA*). No bumps were detectable. CPA not only strongly desensitized the photoreceptor but also changed the waveform of the photoresponse. – C. Semilogarithmic plots of the maximum amplitude of the photoresponse evoked by the step of light *versus* the light intensity. Bathing the photoreceptor with calcium-free saline increased its sensitivity while the addition of CPA desensitized it and shifted the response-intensity curve towards higher intensities by about 2 log units. The photoreceptor was clamped at −68 mV.

dual effect on the light response. While under steady state conditions free cytosolic calcium impairs the generation of single-photon responses, some rapid (probably local) increase in $Ca_i$ appears obligatory to evoke them. Indeed, calcium led to depolarization of the photoreceptor only when pressure-injected (Payne *et al.*, 1986b), not when applied ionophoretically (Lisman and Brown, 1972).

To study adaptation, we analyzed bumps starting 2.5 s after a conditioning (adapting) stimulus, at a time when the intracellular calcium concentration is still elevated above normal (Nagy and Stieve, 1983). Consequently, bumps were light-adapted and their size considerably reduced. Bathing the photoreceptors in low calcium saline did not significantly impair bump adaptation. Earlier studies showed that variation of the external calcium concentration between less than 1 μM and 40 mM only moderately influenced bump adaptation (Stieve and Bruns, 1980, 1983). This indicates that the steady state $Ca_i$ before the conditioning stimulus is of relatively little importance for the process of light adaptation.

On the other hand, buffering the light-induced calcium transient by EGTA injection considerably diminished bump size reduction after the conditioning stimulus. Similarly, injection of calcium buffers impaired light adaptation of macroscopic photocurrents by preventing the increase in cytosolic calcium induced by light or inositol 1,4,5-trisphosphate ($InsP_3$; Lisman and Brown, 1975; Payne *et al.,* 1986a,b). Thus, the transient increase in $Ca_i$ appears to be prerequisite for light adaptation.

*Depleting the internal calcium stores*

Keeping the photoreceptors in low calcium saline for even prolonged periods of time did not desensitize them. Not even several bright flashes, applied in order to deplete the internal stores and thus prevent calcium release, significantly affected the generation of bumps. Light adaptation was reduced but not abolished. It appears that the stores were not completely emptied by the flashes. Alternatively, the mechanism refilling the stores may be very efficient and not disturbed unless the external calcium ions are fully withdrawn or the photoreceptor is intensely illuminated for a long time. Indeed, with repetitive illumination and low or zero extracellular calcium the increase in $Ca_i$ became gradually smaller and eventually almost disappeared (Maaz and Stieve, 1980; Bolsover and Brown, 1985).

Cyclopiazonic acid desensitized photoreceptors within about 60–120 s, which agrees well with the time course of CPA-induced calcium release from stores in HL-60 cells (Demaurex *et al.,* 1992). While bumps were not resolved under these conditions, at high light intensities and physiological external calcium concentrations macroscopic responses with small amplitudes but relatively normal kinetics were evoked. They had a fast transient and a small plateau. This suggests that either the stores were not exhaused, which appears unlikely considering the high CPA concentration used, or that the influx of calcium from the external space was sufficient for response generation and adaptation during the light step. In an earlier study, we estimated that with normal external calcium concentration in some cells up to 60% of the calcium signal monitored by arsenazo was created by calcium influx through "light-activated" plasma membrane channels (Stommel *et al.,* 1996).

In calcium-free saline, the response to a light step after CPA application no longer consisted of a fast transient and plateau but was more or less square. With a saturating light flash under otherwise similar experimental conditions (30 min CPA in calcium-free saline), Ukhanov and Payne (1995) found no detectable increase in $Ca_i$ using fluo-3 as calcium indicator. Yet, they recorded an electrical photoresponse. The largest photocurrent was similar in amplitude (but not kinetics) to the largest photocurrent in normal saline without CPA. Their and our findings contradict the notion that an increase in $Ca_i$ is in any case obligatory on excitation of the photoreceptor. In our opinion this contradiction can be best explained by the assumption that the photocurrent in the ventral photoreceptor contains several components and that one of them does not require a rise in $Ca_i$ for generation. It was suggested that three components ($C_1$-$C_3$) are generated by three parallel transduction cascades (Deckert, Nagy, Helrich and Stieve, 1992). Intracellular application of the calcium chelator BAPTA completely blocked the $C_2$ component in the photocurrent, while the other components were only moderately influenced. The $C_2$ component is thought to include the $InsP_3$-induced calcium release from the internal stores (Contzen *et al.,* 1995). Similarly, only one type of bumps in the ventral photoreceptor, the "standard" or $C_2$ bumps that constitute the macroscopic $C_2$ component (Nagy, Contzen and Stieve, 1993), are influenced by changes in $Ca_i$. The $C_1$ bumps with smaller amplitudes and slower kinetics are virtually insensitive. Their size was not changed either in low external calcium concentration or after the

microinjection of 1.2 mM EGTA (Dorlöchter *et al.*, 1996). Also, the transient and plateau phases in the response to a long stimulus behave differentially to several treatments and were therefore attributed to different mechanisms (Frank and Fein, 1991; Faddis and Brown, 1993). Contzen *et al.* (1995) proposed that the transient is built by the $C_2$ component while the plateau consists of $C_1$ or $C_3$ or a mixture of both. The $C_3$ component has slow kinetics and is activated only at high light intensities in the deeply dark-adapted photoreceptor. It then dominates the photoresponse and determines the maximally possible current amplitude of the photoreceptor (Deckert *et al.*, 1992; Contzen and Nagy, 1996). Thus, we can speculate that when the internal calcium stores were depleted by CPA and no external calcium was available, this component could probably still be activated.

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