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The role of calcium in light adaptation of the crayfish photoreceptor

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The Role of Calcium in Light Adaptation of the Crayfish Photoreceptor

by

Edward M. Son

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ABSTRACT

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As previously shown in many other photoreceptors, during prolonged exposure to light, receptor sensitivity (mv/photon catch) declines and the speed of the response (rate of depolarization and rate of repolarization) increases. The functional significance of internal and external calcium in these changes were examined in the crayfish. We show that the response time characteristics depend significantly on the internal calcium level, whereas sensitivity appears to depend on the extracellular calcium. The effect of BAPTA, a calcium chelator, within the photoreceptor was to slow the response kinetics without changing sensitivity. We also examined the role of potassium channel blockers in accounting for BAPTA's effect. Although these blockers displayed similar effects to that of BAPTA, the data fail to provide irrefutable evidence that calcium works through the activation or the deactivation of K+ conductances alone. Probably (even exclusively), internal calcium also affects the transduction process that precedes the channels openings.
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INTRODUCTION

Light adaptation in vertebrate and invertebrate photoreceptors involves the manipulation of two primary aspects of the visual response: reduction in sensitivity, and reduction in the time course of the response (Fuortes and Hodgkin, 1964; Baylor et al., 1974a; Naka et al., 1987). The first effect has an immediate and useful consequence, providing for a greater dynamic range of response capability to environmental intensity gradients, by adjusting the mean response level in accordance with the mean intensity level. A secondary effect of desensitization is manifested in response to a suprathreshold step input of light. The response displays an initial transient which subsequently decays to some lower steady state level. The functional significance of a reduced time course is increased temporal resolution which enhances motion detection. This is a universal characteristic of light adaptation. The fact that adaptation involves both a change in sensitivity as well as the time course has laid the foundation for an elegant model of transduction in the Limulus (Fuortes and Hodgkin, 1964).

Given the functional importance of light adaptation, the focus of extensive research has been the identity of the intracellular agent and the nature of the control involved in the process of modulation. The recurrent theme is the role of intracellular calcium in sensitivity control, despite the apparently differing mechanisms of transduction in vertebrate and invertebrate photoreceptors.

Transduction

Excitation in the vertebrate photoreceptor (Fain and Matthews, 1990) begins with the absorption of a photon by the photopigment rhodopsin. The resulting change in conformation facilitates binding of rhodopsin to the G protein transducin. Binding alters the conformation of
transducin, and this in turn triggers the exchange of GTP for GDP at the alpha-subunit of the transducin molecule, and the release of the activated subunit T-GTP. T-GTP then binds to and causes the activation of the cGMP phosphodiesterase (PDE), and activated PDE then hydrolyses cGMP at a rate that appears to be diffusion limited.

In the dark, a constant "dark current" is maintained via the influx of Na⁺ ions into the outer segment of the photoreceptor. The ions are expelled by the Na⁺/K⁺ pump located in the inner segment. This current depolarizes the cell and maintains a membrane potential at about -30 to -40 mV (Kaupp, 1986). The decisive experiments of Fesenko and colleagues showed that the application of cGMP to membrane patches of retinal rod outer segments was able to directly regulate the openings of the Na⁺ conductances (Fesenko et al., 1985). The hydrolysis of cGMP therefore leads to the termination of this "dark current"; i.e., the cell hyperpolarizes in response to light.

Once the excitatory cascade has been activated, some process or processes must exist to return the light-activated enzymes to their dark-adapted state when the light has been turned off, and to restore the cGMP concentration to its dark-adapted level. Three such processes have been identified. First, photo-activated rhodopsin is phosphorylated by rhodopsin kinase, a 68 kDa soluble protein. A 48 kDa protein, now generally referred to as arrestin, binds to phosphorylated rhodopsin and sterically hinders the binding of rhodopsin to transducin. Second, the active transducin subunit, T-GTP, spontaneously hydrolyses GTP to GDP. This forms T-GDP, which dissociates from the gamma-subunit of the PDE. T-GDP then recombines with the T (beta) and T (gamma) subunits, and the gamma-subunits of the PDE reassociate with PDE (alpha) and PDE (beta) to return the
PDE to a low, basal activity. Third, the cGMP concentration is returned to its dark-adapted level by a calcium regulated enzyme guanylate cyclase.

In contrast with vertebrates, for which the transduction mechanisms are reasonably clear, much less is known about the molecular mechanisms of invertebrate phototransduction (Rayer, Naynert, and Stieve, 1990). The identity of the terminal internal messenger is still unknown and several intermediate steps of the enzyme cascade have not been determined.

There is evidence that light-activated rhodopsin coupled to a G-protein is also involved in invertebrate vision. This is indicated by the observation that guanine nucleotide analogues or fluoride and vanadate induce so-called "quantum bumps" in the dark (miniature depolarizations thought to be caused by single photon captures) resembling those produced by light. One target of the invertebrate photoreceptor G-protein is phospholipase C, a phosphodiesterase, which catalyses the hydrolysis of phosphatidyl-inositol-bisphosphate (PIP2) into inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (Devery et al., 1987; Baer and Sibil, 1988). IP3 is released into the cytoplasm. It is proposed as a candidate for a second messenger in visual transduction. Since all of these steps are demonstrated to be activated by light, the results strongly suggest that the flow of information in invertebrate phototransduction is from photoexcited rhodopsin to G-protein, and from G-protein to phospholipase C, which causes an increase in the intracellular level of IP3 concentration, and an IP3-induced calcium release from the intracellular cisternae (Brown and Rubin, 1984; Payne et al., 1986). It is hypothesized that stimulation by light leads to the calcium activated increase in Na⁺ conductance and the consequent depolarization of the cell, which contrasts with the light activated decrease in conductance and hyperpolarization of the cell in the vertebrate receptor.
Increasing intracellular levels of IP3 in *Limulus* ventral photoreceptors causes the release of internal calcium stores (Rayer, Naynert, and Stieve, 1990). This has been demonstrated by injection of IP3 into *Limulus* ventral photoreceptors that had previously been loaded with aequorin, a phosphoprotein, which luminesces in the presence of free calcium (Brown and Blinks, 1974). IP3 causes an increase in aequorin luminescence as does illumination, and therefore calcium is proposed as the terminal internal messenger.

IP3 induced excitation and desensitization are abolished by prior injection of calcium chelators such as EGTA or BAPTA into the cell, which suggests that both excitation and adaptation by IP3 are mediated via an increase in intracellular calcium. However, EGTA injection does not block the response induced by light (Payne, Corson, and Fein, 1986). Therefore light may act through another pathway to excite the cell.

Experiments by Johnson et al. (1986) have shown that injection of cGMP into selective rhabdomeric regions of the *Limulus* ventral photoreceptors produces excitation with a similar time course of light induced depolarizations. These cGMP induced potentials exhibited identical reversal potentials to those of the light responses, and light stimulation increased the level of cGMP in the giant squid receptors. Moreover, cGMP-induced excitation was not blocked by the presence of calcium chelators. These evidences point to the possibility of an alternative pathway to excitation in the *Limulus*.

**Light adaptation**

In the vertebrate rod, the current response to a dim flash begins with a delayed sigmoidal onset, rises to a rounded peak and then slowly decays to the baseline. The time-to-peak varies with temperature and between
species, eg. 0.6-4 s in amphibians (Baylor et al., 1979a) or approximately 250 ms in mammals (Nunn and Baylor, 1982). With increasing flash intensity the response increases up to a saturating level at which the "negative" photocurrent totally annihilates the dark current. The voltage responses at saturating intensities show a rapid initial peak which relaxes to a lower plateau, followed by a decay. There is almost no variation in time course with intensity (Baylor et al., 1979b).

Light adaptation in the vertebrate rod is accompanied by an acceleration in the rate of decay of the response. It is as if responses in the presence of an adapting light are turned off prematurely, and the stronger the adaptation, the earlier this decay process begins. Baylor and Hodgkin (Baylor and Hodgkin, 1974b) proposed that the acceleration of decay was the basic cause of adaptation and was directly responsible for all of its manifestations, including the decrease in sensitivity.

The story appears to be a little more complicated in the cones, where changes in the initial time course of the response are also observed with light adaptation (Naka et al., 1987; Baylor et al., 1974a). In the presence of a higher mean irradiance, causing light adaptation, the latency and the rise times of the response were also seen to diminish. Apparently, adaptation in these cells involve additional mechanisms to the effects seen on the decay.

Changes in the concentration of cytoplasmic free calcium have been shown to affect the light responses of both rods and cones (Miller, 1981); and many of these effects are now known to be caused not by a direct effect of calcium on the light-dependent conductance but rather by indirect effects on one or more of the enzymatic steps that lead to changes in cGMP levels. Furthermore, light produces a change in [Ca$^{2+}$]i. Calcium accounts for
10-15% of the dark current, so that for an amphibian rod, about $10^7 \text{Ca}^{2+}$ ions per second flow continuously into the outer segment (Fain and Matthews, 1990). Light decreases this influx but appears to have no direct effect on Ca$^{2+}$ efflux mediated by the Na$^+$/Ca$^{2+}$,K$^+$ counter-exchanger (Nakatani and Yau, 1988). Therefore light should decrease the Ca$^{2+}$ concentration, which should vary monotonically with background intensity. Light induced decreases in [Ca$^{2+}$]$_i$ have recently been measured using both aequorin and fura-2 (Ratto et al., 1988).

In the invertebrates the membrane potential in dark is primarily determined by the potassium gradient, around -30 to -70 mV depending on the species. Stimulation by light causes a depolarization of the cell caused mainly by Na$^+$ influx. A dim light source gives rise to small responses (quantal bumps) with a latency of about 200 ms, a rise time of about 30 ms, and an exponential decay with a time constant of about 40 ms. In contrast to the vertebrate rod, the invertebrate cell exhibits a high variability in the latency, amplitude, and time course of the quanital bumps (Stieve, 1985).

According to the adapting bump model (Dodge, Knight, and Toyoda, 1968), adaptation by light causes a shortening of the latency and time course of the response (Stieve, 1985; Howard et al., 1984) as well as a decrease of response amplitude. These phenomena can be explained in terms of the change in the underlying quanital bump characteristics as a result of adaptation (Wong 1978). Light adaptation decreases the amplitude and the time course of the quanital bumps which make up the total response, resulting in the observed changes in macroscopic response characteristics.

In the Limulus ventral photoreceptors, a drop in sensitivity of the cell caused by an adapting stimulus coincided with a rise in intracellular calcium concentration, as measured by Ca-selective microelectrodes (Levy
and Fein, 1985). A 100-fold desensitization was associated with a 2.5-fold increase in $[\text{Ca}^{2+}]_i$. Furthermore, injection of EGTA markedly decreased the light-induced $[\text{Ca}^{2+}]_i$ rise and increased the time to peak of the light response, but did not alter the resting $[\text{Ca}^{2+}]_i$, which suggests that the time to peak is affected by a change in the capacity to bind calcium and not by resting $[\text{Ca}^{2+}]_i$.

**External Calcium**

It has been shown that the vertebrate response is virtually independent of intracellular stores of calcium. Excitation in cells depleted of intracellular calcium stores still persisted (Nicol et al., 1987). When intracellular levels of calcium were maintained at a low level by removing all extracellular calcium and substituting external sodium with Li$^+$ or guanidium- so as to hinder the Na$^+$/K$^+$/Ca$^{2+}$ pump, the responses had prolonged decay compared to responses in normal conditions (Fain and Matthews, 1990). The initial portion of the response was unaffected; however every manifestation of light adaptation was eliminated. Photoreceptors behaved as linear photon detectors, and the responses to single photons summed in a manner that is invariant with the intensity and duration of illumination.

In the *Limulus* both light adaptation and the rate of dark adaptation after exposure to bright light were strongly dependent on external calcium (Stieve et al., 1984; Stieve, 1981). In fact, intracellular calcium concentration was directly proportional to external calcium level (Levy and Fein, 1985). The effect of $[\text{Ca}^{2+}]_o$ in dark adapted cells were minimal, however light adaptation (shift in sensitivity) was greater in the presence of external calcium. The rising phase of the response was minimally affected by the absence of calcium, but the decline was prolonged (Stieve et al., 1983). To
a steady state light the transient portion of the response was reduced, while the plateau was enhanced.

Similarly in the crayfish (Stieve and Hanani, 1976), low external calcium affected primarily the decay portion of the response. It also increased the overall amplitude, reduced the amount of sensitivity change as a result of light adaptation, and accelerated the rate of dark adaptation from light adapted states. The results in barnacle lateral photoreceptors were similar.

In the eye of Drosophila (Ranganathan et al., 1991) both the rate of activation and deactivation (decay) of the inward currents to light appeared to depend on external calcium, the rates slowing significantly with the reduction in external calcium. (It appears that the amplitude also decreased.) Furthermore, these results are supported by the existence of an inaC mutant defective in the calcium regulated rapid deactivation process; responses in these mutants were protracted and slowly decaying in time. The locus of the genetic mutation was later shown to be the site for an eye protein kinase C involved with the calcium activated deactivation (Smith et al., 1991). Apparently, a phosphorylation step intervenes calcium's action on the cell.

**Calcium chelators**

The first clear evidence that a change in [Ca$^{2+}$]$_i$ might be responsible for light adaptation in vertebrate photoreceptors came from experiments in which the Ca$^{2+}$ chelator BAPTA was incorporated into isolated rods (Torre, Matthews, and Lamb, 1986). (BAPTA possesses a faster chelating action than EGTA and therefore is a more suitable buffer for the light induced changes in internal calcium.) It produced a pronounced retardation in the falling phase of the response, allowing the response to rise further and increasing the sensitivity. How can these effects of BAPTA be inter-
preted? Suppose that under normal conditions the waveform of the light response is the result of two processes, one, the activation of the PDE produced by the excitatory cascade, and two, some other process that is triggered by a decrease in [Ca$^{2+}$]$_i$ and that is responsible for the recovery of cGMP concentration to its dark-adapted level. If the buffering capacity of the rod for [Ca$^{2+}$]$_i$ were increased, this second process would be greatly retarded (the salient feature in this scenario is BAPTA’s ability to prevent sudden changes in internal calcium milieu, and the implication that the important factor in light-dependent deactivation is the transient decrease in calcium). The waveform of the light response would then primarily reflect the time-course of the first process, the excitatory cascade.

Since neither background light nor strategies that slow down changes in [Ca$^{2+}$]$_i$ seem to have much effect on the latency or initial time-course of the light response, it is unlikely that [Ca$^{2+}$]$_i$ produces a significant alteration in the velocity of any of the steps that lead directly to PDE activation and the decrease in cGMP. (However this does not preclude the possibility that [Ca$^{2+}$]$_i$ could affect the life-times of one of the intermediates of the excitatory cascade.)

An alternative hypothesis is that the decrease in [Ca$^{2+}$]$_i$ produced by illumination causes an increase in the rate of activity of the cyclase (Cohen et al., 1978; Forti et al., 1989). A mechanism of this nature would accelerate the time-course of decay of the receptor response by increasing the rate with which the cGMP concentration is returned to its steady-state value.

It is worth mentioning that although a change in [Ca$^{2+}$]$_i$ is necessary for light adaptation, it may not be sufficient; that is, merely changing the [Ca$^{2+}$]$_i$ may not suffice to alter sensitivity. The experiments of Detwiler
and his colleagues (Sather et al., 1988) seem in fact to indicate that, in addition to changes in $[Ca^{2+}]_i$, light stimulation must also be present. Treatments that alter $[Ca^{2+}]_i$ have markedly different effects on sensitivity depending upon whether or not light is present.

In the *Limulus*, iontophoretic injection of EGTA (Lisman and Brown, 1975; Levy and Fein, 1985), a calcium chelator, into the ventral photoreceptor caused 1) a decrease in sensitivity, as evidenced by a higher test flash intensity needed to produce the same amplitude criterion response before the injection; 2) a prolongation of the time to peak of responses to dim test flashes; 3) a longer duration of the responses to brief flashes after EGTA injection. These effects suggest that calcium plays an important role in both excitation and adaptation. The decrease in sensitivity and the longer time to peak can be accounted for by the buffering power of EGTA on calcium, an agent thought to be important in invertebrate transduction. However, calcium also appears to play a role in the desensitization of the cell, since EGTA prevented the rapid deactivation of the response. Additional effects include- 1) EGTA injection prevented the decline from transient to plateau of steady state responses; 2) after injection the response-intensity curves tended toward linearity; 3) it decreased the light-induced changes in sensitivity. That is, EGTA minimized all manifestations of light adaptation.

The mechanisms of light adaptation and transduction in the vertebrate receptors are more or less conclusive; however, the observations on the role of calcium, particularly extracellular calcium, in the invertebrate photoreceptor is much more varied, if not inconsistent. The issue is clouded by the fact that calcium appears to play a dual role of the adapting factor as well as the excitatory agent. Here it is shown that in the crayfish,
the role of external and internal calcuums are functionally distinct, and that light adaptation is most probably mediated by calcium's modulatory role on the transduction process.

**METHODS**

The eyestalk of the crayfish (*Procambarus Clarkii*) were excised from the animal and mounted in a small chamber containing cooled, oxygenated Van Harreveld's solution (14°C) which has the following composition: Na+ 207.3, K+ 5, Ca²⁺ 14, Mg²⁺ 3, Cl⁻ 244, HCO₃⁻ 2.3 mM. The photoreceptors were illuminated by a halogen-lamp light source which was passed through an electromechanical shutter, a condensing lens, and a graded neutral filter. Recordings were made using microelectrodes of 70-100 MΩ resistance.

The chemicals were prepared in saline (50 mM BAPTA) and were either injected iontophoretically (using current of -.7 nA for 10-20') or were perfused (at ~5 ml/min) in the saline bath using a perfusion system, at concentrations of 5 mM 4-AP & 5 mM TEA, 40 mM Ca²⁺, or 0 mM Ca²⁺. In preparing the differing concentrations of external calcium, calcium was added or removed and proper osmolarity of the saline solutions maintained by regulating the NaCl equivalents.

**RESULTS**

The results can be divided into three categories; the effect of external calcium, BAPTA injection, and potassium channel blockers (4-AP & TEA) on the sensitivity and time course of the response and its adaptation by light.
In the dark, external calcium had little or no effect on the time-to-peak (time from the onset of stimulus to the peak of the response); figure 1 shows the responses before and after exchanging external bath solution from 0 calcium to normal calcium. While sensitivity is visibly reduced, the time course of the response remains essentially the same. Figure 2a shows the averages of six and four runs, where external bath is changed from calcium free to normal calcium (CaN) or to 40 mM calcium (Ca40), which is three times the normal calcium load. The time-to-peak is minimally affected by the exchanges to calcium containing baths. Observation of figure 2b, however, shows that sensitivity is reduced rather dramatically by the presence of external calcium (Ca40 and CaN)- significant within 90%.

**Fig. 1.** Responses taken after exchanging external bath solution in dark from no Ca to 40 mM Ca. The amplitude of the response is significantly reduced. Trace CF, dark adapted control in Ca free; trace C40, after exchange of solution. Responses to 20 ms light pulses, at log of intensity equal to -4.5 (maximum intensity being 1). Averages of 5 responses each.

**Fig. 2A.** The time-to-peak of the response were measured for the various given conditions in dark before and after the treatments. Bapta, 50 mM BAPTA; Ca40, from Ca free solution to 40 mM calcium; CaN, from Ca free solution to normal saline; KB, from control to 5 mM TEA & 5 mM 4-AP. Treatments were carried out as discussed in METHODS. Bapta, n of 7; Ca40, n of 6; CaN, n of 4; KB, n of 6. * means significant within 95%, using the t test.
The amplitude reductions produced by increasing external calcium from 0 to 13.5 or 40 mM are comparable in degree, despite the fact that 40 mM is thrice the normal concentration. These results show a qualitative agreement with previous results in the crayfish, and also in the *Limulus*, the Barnacle, and the vertebrate rods and cones. The vertebrate receptors, however, showed a dramatic dependence on the external calcium, beyond those of the invertebrate receptors (Fain and Matthews, 1990). The Drosophila, on the other hand, also exhibited a dependence of the "activation" rate on external calcium.

Introducing external calcium, the decay rates were also enhanced, qualitatively (data not shown), in agreement with previous results in the crayfish and other systems.
**Fig. 3A.** Response taken after light adaptation in Ca free bath (prepared as in METHODS). The amplitude of the response is significantly reduced; the peaktime is also affected a little. Trace D, dark adapted control in Ca normal; trace L, after adaptation. Responses to 20 ms light pulses, at log of intensity equal to -5 (maximum intensity being 1). Averages of 10 responses each.

**Fig. 3B.** Responses taken after light adaptation in normal Ca bath. The amplitude of the response is significantly reduced; the peaktime is also affected. Trace D, dark adapted control in Ca normal; trace L, after adaptation. Responses to 20 ms light pulses, at log of intensity equal to -2.5 (maximum intensity being 1). Averages of 1 each.

External calcium, however, appears to play no significant role on the operation of light adaptation on the response features. Figure 3a shows light adaptation in calcium free bath, and figure 3b in normal calcium bath. The primary effects are on the sensitivity of the responses. The next two figures describe the ensemble of averages for such treatments on the time-to-peak and the amplitude of the response (see fig's 4a & 4b- CaF and CaN).
Fig. 4A. The time-to-peak of the response were measured in the various given conditions before and after light adaptation. postB, in 50 mM BAPTA; postKB, in 5 mM TEA & 5 mM 4-AP; CaF, in Ca free solution; CaN, in normal Ca bath. Treatments were carried out as discussed in METHODS. postB, n of 5; post KB, n of 5; CaF, n of 13; CaN, n of 13. * means significant within 95% using the t test.

Fig. 4B. The amplitude of the response were measured in the various given conditions before and after light adaptation. postB, in 50 mM BAPTA; postKB, in 5 mM TEA & 5 mM 4-AP; CaF, in Ca free solution; CaN, in normal Ca bath. Treatments were carried out as discussed in METHODS. postB, n of 6; post KB, n of 5; CaF, n of 14; CaN, n of 13. * means significant within 95% using the t test.

Figures 4a and 4b describe the effects of light adaptation in the presence of the given conditions. The purpose of this study was to examine the effects of these conditions on the normal operation of light adaptation. The light adapted peaktime is slightly quicker with external calcium (CaN-normal calcium vs. CaF- calcium free)- see figure 4a, but the sensitivity declines are virtually indistinguishable, with or without the calcium’s presence outside the cell- (CaN and CaF) figure 4b. These results, particularly
on sensitivity, are in direct confrontation with effects seen in other animals. It should be pointed out that the adapting intensities used in these experiments, had almost no effect on the time course of the control (CaN, fig. 4a or 7b later); higher intensities in previous works on the crayfish, enough to produce alterations of 250 fold in sensitivity, also diminished the rise time of the response by 50% (Glantz, 1991). Perhaps, stronger adapting intensities would have elicited differential sensitivity changes as well. Perhaps, also, longer equilibration time of exchanged solutions would have effected stronger changes, by allowing more calcium seepage into the cell (equilibration times were usually 10 minutes or longer).

BAPTA’s effects in crayfish were similar to the influence of EGTA injections in the Limulus (fig 5). The sensitivity diminished minimally while BAPTA greatly prolonged the time course of the response (in the figure from D, the dark adapted control, to B, after BAPTA in dark). (Also observe average changes in peak time and amplitude as a result of BAPTA treatment- "Bapta" in figures 2a and 2b.) Examination of response curves in figure 5 indicates that decay time is particularly exaggerated post-BAPTA (also see figure 6 for an average result of BAPTA on the decay time constant).
Also examining the effects of light adaptation in the presence of the buffer, light's potency appears to be somewhat enhanced, giving greater percentage reduction in peaktime and amplitude (fig's 4a & 4b- condition postB) compared to control (normal calcium- CaN). However, a significant proportion of this change is probably due to the fact that in the BAPTA condition, as opposed to the CaN condition, the cell exists "as if" in a state of higher dark adaptation: it was shown that BAPTA lengthens the time course- a property associated with a state of dark adaptation; and with BAPTA, the sensitivity did not decline, whereas with CaN, the sensitivity was reduced noticeably. Thus, the same adapting light is liable to have a greater effectiveness in the BAPTA case.

However, when one calculates the "absolute" changes induced by light, i.e. normalized relative to the original dark-adapted control, comparison histograms in figures 7a and 7b show that BAPTA actually tended to minimize light adaptation as compared with CaN. This minimization was rather significant for the peaktime and less for the sensitivity (see the levels of the gray bars which indicate the effects of light adaptation in the pres-
ence of the given conditions, normalized to the black bars— the controls— rather than to the striped bars— the post-treatment conditions).

**Fig. 7A.** Comparison histograms for amplitudes normalized with respect to control (before treatments in dark). The black bars were given arbitrary values of 100; striped bars represent the amplitudes after the treatments in the dark (normalized to control); gray bars are values after light adaptation. These values were calculated using data from previous histograms. CaN, normal calcium bath; Bapta, 50 mM BAPTA; KB, 5 mM TEA & 5 mM 4-AP. Treatments were carried out as discussed in METHODS.
The effects of $K^+$ channel blockers (KB) appears to only mimic BAPTA at first glance (fig's 2a, 2b & 4a, 4b- conditions KB and postKB). However, the delay on decay times was weaker with the $K^+$ blockers than with BAPTA (see figure 6; also see figure 9b later, the striped bars); and if the response waveforms are inspected, the differences in decay rates are quite apparent (fig 8 vs. fig 5); the lengthening of the decay time is much more pronounced with BAPTA then with $K^+$ blockers.
Fig. 8. Responses taken before and after K blockers, TEA & 4-AP (applied as described in METHODS) and after light adaptation, still blocked. Trace D, dark adapted control; trace B, after K blockers; trace LB, light adaptation in blocked state. The effect of the blockers is primarily on the time course, and specifically on the decay portion of the response. Light adaptation is still significant, despite the blockers. Responses to 20 ms light pulses, at log of intensity equal to -1 (maximum intensity being 1). Averages of 5 responses each.

Again in figure 9a which show the average effect of light adaptation on the decay for K+ blockers and BAPTA, the apparent greater percentage change with BAPTA can be accounted for by BAPTA’s originally higher sensitiveness (for decay rates, at any rate); difference in final levels in either case is insignificant (see fig 9b).

Fig. 9A. The decay times of the responses (single exponential time constant of decay after the peak) were measured in the various given conditions before and after light adaptation. CaN, normal calcium; KB, in 5 mM TEA & 5 mM 4-AP; Bapta, in 50 mM BAPTA. Treatments were carried out as discussed in METHODS. CaN, n of 9; KB, n of 2 Bapta, n of 2. * means significant within 95%, *** significant within 80%, using the t test.
Fig. 9B. Comparison histograms for decay times after the peak in response (single exponential time constant) normalized with respect to control (before treatments in dark). The black bars were given arbitrary values of 100; striped bars represent the decay times after the treatments in the dark (normalized to control); gray bars are values after light adaptation. These values were calculated using data from previous histograms. CaN, normal calcium bath; Bapta, 50 mM BAPTA; KB, 5 mM TEA & 5 mM 4-AP. Treatments were carried out as discussed in METHODS.

Upon examination of the comparison histograms, light adaptation on peaktimes also appear to be inhibited for the K+ blockers compared with the control (CaN) (fig 7b), as it was with BAPTA. This tendency is reflected in light's effect on the decay times as well. Inspection of figure 9a might imply that adaptation showed a relatively weak differential for the given two conditions (BAPTA and KB), compared with the no treatment (CaN). However, figure 9b (the gray bars) shows that in terms of absolute levels, these treatments, especially BAPTA, were successful in curbing light's effect on the decay as well. The apparent effectiveness of TEA and 4-AP in blocking the effects of light adaptation will be discussed later.

DISCUSSION

These results indicate that external calcium, or the lack thereof, has a distinct effect on the response characteristics of the crayfish (dramatic effect on the sensitivity without coequal effect on the time course).
Moreover, manipulation of the level and effects of internal calcium by BAPTA are functionally separable from the manipulation by external calcium loads, since these two manipulations did not produce comparable effects. To sum, in the former case, increase in external calcium decreased sensitivity only, while in the latter, increase in internal calcium (BAPTA’s antithesis) would have decreased the time course only. One implication is that external calcium has a relatively limited access into the cell.

In other invertebrate preparations, for eg. the Drosophila (Ranganathan et al., 1991), increase in intracellular calcium is primarily the consequence of calcium influx during the light response. In the ventral photoreceptor of the *Limulus*, the increase in intracellular calcium during the response is primarily due to release of intracellular calcium stores (Stieve and Hanani, 1976). In this respect, the crayfish receptors may be similar to the *Limulus*.

One plausible scenario is that outside calcium directly manipulates the receptor membrane properties externally (such as the input resistance), affecting primarily the amplitude of the response (Stieve and Hanani, 1976), while BAPTA affects the internal workings of the transduction process. (Laboratory measurements of input resistance (Rn) at membrane resting potential showed that Rn did not change appreciatively with external calcium, however.) It has also been found that external divalent cations have varying degrees of "flickering blockage" on cationic conductance in membrane patches of the NMDA receptor-channel (Ascher and Nowak, 1987) and the vertebrate outer-segment light channel (Haynes, Kay, and Yau, 1986; Hagins and Yoshikami, 1974; Bastian and Fain, 1982). It is hypothesized that the mechanism of block is associated with dehydration
times of permeants involved in cationic conductances and/or different binding strength for variable ionic species within the channel itself.

It is also possible that the lack of effect on the amplitude of the response merely indicates that these cells had reached the limits of their sensitivity profile (they were dark adapted beyond 30'). Thus, the cells were able to respond with decrease in sensitivity to external calcium seeping in, but could not answer with proper increase in sensitivity to BAPTA's buffering action. The kinetics profile, on the other hand, may possess an entirely different range of calcium sensitivity, which could account for the different behavior of the time course in response to BAPTA and external calcium loads. In support of this conjecture, figure 7a indicates that BAPTA may be inhibiting light's efficacy on sensitivity. Thus, the uncoupling of calcium and sensitivity seen here, and also in the Limulus (see text later), may apply only under special conditions.

Whether or not the sensitivity capacity of the cells were overtaxed, the available conclusion to be drawn from these experiments is that BAPTA appears to affect only the kinetics of the response. The rate of rise as well as the rate of decay are both prolonged as a result of BAPTA injection; and BAPTA is able to inhibit the decrease in time course (both peak time and decay time) seen with light adaptation. These in turn support the conclusion that calcium is important for the regulation of the kinetics of phototransduction. In as much as light adaptation in the crayfish involves reduction in the time course, calcium is an agent of adaptation in the crayfish.

The implication is that changes in the kinetics of transduction may be functionally separated from a concomitant alteration in the sensitivity or amplitude of the response. Previously, all modeling of the invertebrate
transduction was based on the functional interdependence of the two properties of transduction— for eg., in the *Limulus* (Fuortes and Hodgkin, 1964). Moreover, models implying sensitivity change by a more rapid decay (vertebrate rod- Baylor and Hodgkin, 1974b), which imply a close association of a change in sensitivity and a change in the decay rate, likewise becomes inappropriate here due to the BAPTA results.

Nonetheless, there are few exceptions in the literature which also refute the calcium-sensitivity interdependence. It was mentioned in the INTRODUCTION that vertebrate receptors responded quite differently to changes in internal calcium whether or not light was present. Previous experiments controlling for the quantitative relationship between sensitivity and intracellular calcium levels have determined that calcium levels alone could not account for the decrease in sensitivity seen with bright orange light adaptation in the crayfish (Cummins and Goldsmith, 1985). Moreover, in the *Limulus* itself, it was shown that under certain conditions (extreme low [Ca$^{2+}$]$_i$ load) it is possible to uncouple [Ca$^{2+}$]$_i$ and sensitivity (Levy and Fein, 1985).

In the experiments described by Cummins and Goldsmith, a concern that they dealt with was the effect of a lingering depolarizing-after-potential produced by a strong adapting stimulus. The amplitudes of additional responses superimposed over a slowly decaying maintained depolarization were reduced; a case for non-linear summation (Glantz, 1971a, 1971b). This effect could account for one possible mechanism of light adaptation, even in the absence of elevated internal calcium levels. In the experiments described above, it was seen that desensitization was apparent even after mathematically accounting for the effects of the after-potential. Moreover, in our own observations, none of the treatments led to any noticeable and
consistent effect on the resting membrane potential which could account for the depolarizing-after-potential, even though desensitization was evident in the absence of an after-potential.

One caution should be observed however; in the *Limulus* prep, control injections of mannitol also tended to prolong the time course of the response without affecting its sensitivity (Lisman and Brown, 1975). Therefore, conditions other than the absorption of calcium could be responsible for BAPTA's effect seen on the kinetics, and the results may be (though unlikely) entirely accidental. In defense, it should be pointed out that control injections (performed on the crayfish) of various cationic species (K⁺, Ca²⁺, and Na⁺), though injected under qualitatively different contexts (i.e., in series of short pulses rather than over extended periods, and using much higher currents), produced no such comparable effects on the time course of the response (data not shown).

The Bapta-elicited decrease in sensitivity observed in most cases (in a few cases the sensitivity remained unchanged) is rather minimal. Discounting above qualifications, it appears that variations in intracellular calcium is not sufficient for sensitivity control, and perhaps some other light-induced factor(s) are necessary or mediates changes in sensitivity. This supposition is supported by the fact that light induced desensitization still occurred in the presence of the BAPTA. However, it is possible that BAPTA's action was overwhelmed by the light induced rise in intracellular calcium. The fact that BAPTA's original effects on the time course could be partially reversed by light supports this.

The small reductions in sensitivity caused by BAPTA can be explained on the basis of calcium's probable role in mediating transduction. The fact that the light response was not annihilated by BAPTA also implies
that a rise in intracellular calcium cannot be an absolute requirement for transduction. These implications are in accordance with data seen in the *Limulus* (Lisman and Brown, 1975).

One possibility considered was that calcium, or its lack, effected the response through calcium modulation of K⁺ currents, particularly the two principal currents involved in shaping the response. In some preparations (Chinn and Lisman, 1984; Brown and Brown, 1973) intracellular calcium activates a potassium current (molluscan somma, neural tissues, and Aplysia giant neuron). However, in the Aplysia, the response to light hyperpolarizes, and the K⁺ activation is small and slow (about 400 ms). In the *Limulus* (Pepose and Lisman, 1978), it has been shown that light activated increase in internal calcium deactivates a slow process (the delayed rectifier- which is a K⁺ current). The effect of this deactivation of an "outward" current is to compensate for a reduction in the "inward" current that occurs in steady state light condition. This compensation maintains a steady state of depolarization to a constant light; otherwise the plateau response would drift slowly in time. Pepose and Lisman showed that the injection of TEA, a K⁺ channel blocker, into the *Limulus* photoreceptor mimicked the effects of EGTA injection. The decay phase was prolonged, and the response showed an apparent lack of desensitization. However, the underlying mechanisms were different; in the case of TEA injection, the K⁺ channels were blocked, the load conductance steadily decreased, and therefore a decreasing Na⁺ current was sufficient to maintain the depolarization, whereas in the case of EGTA injection, Na⁺ influx remained large due to a true lack of desensitization, and this effect was able to override the increasing K⁺ channel conductance. The decreased load conductance as a result of K⁺ channel blockage could also explain the effect of 4-AP and
TEA on the increased peaktime seen in the crayfish. The decrease in sensitivity cannot be explained this way however.

It was interesting to observe the effects of blocking the two primary $K^+$ conductances involved in a light response. By blocking them, it was shown that the expression of light adaptation could be inhibited, thereby mimicking the lack of desensitization seen with BAPTA.

The overall results indicate that the physiology of the crayfish receptor is similar to that of the *Limulus*. The response time characteristics show a profound dependence on the internal calcium milieu, and calcium probably plays a significant role on both the excitation and adaptation of the transduction process. The functional separation of sensitivity change and kinetics change was quite apparent, however the role of calcium on sensitivity is rather unclear. The results of $K^+$ channel blockers, though similar to that of BAPTA, fail to provide irrefutable evidence that calcium mediates its effects through the activation or the deactivation of $K^+$ currents. The more probable target of calcium's action is the transduction process that underlies the light response.
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