Evidence for Electrogenic Na^+/Ca^{2+} Exchange in *Limulus* Ventral Photoreceptors

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ABSTRACT The Ca^{2+} indicator photoprotein, aequorin, was used to estimate and monitor intracellular Ca^{2+} levels in *Limulus* ventral photoreceptors during procedures designed to affect Na^+/Ca^{2+} exchange. Dark levels of $[Ca^{2+}]_i$ were estimated at 0.66 ± 0.09 μM. Removal of extracellular Na^+ caused $[Ca^{2+}]_i$ to rise transiently from an estimated 0.5–0.6 μM in a typical cell to ~21 μM; $[Ca^{2+}]_i$ approached a plateau level in 0-Na^+ saline of ~5.5 μM; restoration of normal $[Na^+]_o$ lowered $[Ca^{2+}]_i$ to baseline with a time course of 1 $\log_{10}$ unit per 9 s. The apparent rate of Na_o^+-dependent $[Ca^{2+}]_i$ decline decreased with decreasing $[Ca^{2+}]_i$. Reintroduction of Ca^{2+} to 0-Na^+, 0-Ca^{2+} saline in a typical cell caused a transient rise in $[Ca^{2+}]_i$ from an estimated 0.36 μM (or lower) to ~16.5 μM. This was followed by a decline in $[Ca^{2+}]_i$ approaching a plateau of ~5 μM; subsequent removal of Ca_o^{2+} caused $[Ca^{2+}]_i$ to decline slowly (1 log unit in ~110 s). Intracellular injection of Na^+ in the absence of extracellular Na^+ caused a transient rise in $[Ca^{2+}]_i$ in the presence of normal $[Ca^{2+}]_o$; in 0-Ca^{2+} saline, however, no such rise in $[Ca^{2+}]_i$ was detected. Under constant voltage clamp (−80 mV) inward currents were measured after the addition of Na_o^+ to 0-Na^+ 0-Ca^{2+} saline and outward currents were measured after the addition of Ca_o^{2+} to 0-Na^+ 0-Ca^{2+} saline. The results suggest the presence of an electrogenic Na^+/Ca^{2+} exchange process in the plasma membrane of *Limulus* ventral photoreceptors that can operate in forward (Na_o^+-dependent Ca^{2+} extrusion) or reverse (Na_i^+-dependent Ca^{2+} influx) directions.

INTRODUCTION

Na^+/Ca^{2+} exchange across plasma membranes is important in many cell types for maintaining a low level of intracellular free calcium ions, Ca_i^{2+}, and creating a large transmembrane electrochemical energy gradient for Ca^{2+} (Ca^{2+} gradient). By this process, Ca^{2+} is extruded from the cell against its energy gradient as Na^+ enters the cell down its energy gradient (Reuter and Seitz, 1968; Baker et al., 1969; Blaustein and Hodgkin, 1969). In several cell types, Na^+/Ca^{2+} exchange has been shown to generate a membrane current, a feature that arises from the stoichiometry of Na^+

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J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/89/03/0473/20 $2.00
Volume 93 March 1989 473–492

entry to Ca^{2+} export. For example, in squid giant axon three or more Na^+ enter for each Ca^{2+} extruded (Mullins and Brinley, 1975; Blaustein, 1977), resulting in a net inward current. In some systems (DiPolo, 1979; Mullins and Requena, 1981; DiPolo and Beauge, 1987), Na^+/Ca^{2+} exchange can be reversed, that is, in addition to $[Na^+]_o$-dependent Ca^{2+} extrusion (forward exchange), $[Na^+]_i$-dependent Ca^{2+} entry (reverse exchange) can occur when the transmembrane electrochemical energy gradient for Na^+ is reversed to favor Na^+ entry.

Na^+/Ca^{2+} exchange appears to be present in a number of photoreceptor preparations (Lisman and Brown, 1972; Bader et al., 1976; Bastian and Fain, 1982; Yau and Nakatani, 1984; Minke and Tsacopoulos, 1986; Schnetkamp, 1986; Hodgkin et al., 1987). In *Limulus* ventral photoreceptors, Na^+/Ca^{2+} exchange was proposed by Lisman and Brown (1972) who injected Na^+ into the cytosol of a photoreceptor and observed a $[Ca^{2+}]_o$-dependent reduction in light sensitivity that resembled the effects of raising $[Ca^{2+}]_i$. Brown and Mote (1974) found a similar $[Ca^{2+}]_o$-dependent desensitization resulting from Na_o^+ removal, and Waloga et al., (1975) reported that a $[Ca^{2+}]_i$ rise could be detected following procedures designed to reduce the transmembrane Na^+ gradient.

The universal requirement for Ca^{2+} regulation in neurons underlies the significance of Na^+/Ca^{2+} exchange. Intracellular free Ca^{2+} is involved in or responsible for regulation of physiological processes in virtually every excitable cell. Moreover, Ca_i^{2+} may influence several different time-dependent intracellular processes in different ways within the same cell at the same time. Therefore, the spatial and temporal profiles of $[Ca^{2+}]_i$ must be extremely important cellular features, requiring precise control. In the *Limulus* ventral photoreceptor, light initiates a complex series of biochemical reactions, resulting in a depolarizing receptor potential (Millecchia and Mauro, 1969*a*). Intracellular Ca^{2+} appears to play a role in this process (Bolsover and Brown, 1985; Payne et al., 1986). Light also triggers the mobilization of Ca^{2+} from intracellular stores, raising $[Ca^{2+}]_i$ levels significantly (Brown and Blinks, 1974). A consequence of raising $[Ca^{2+}]_i$ is a transient reduction in the electrical responsiveness of the cell to subsequent illumination (Lisman and Brown, 1972; Brown and Lisman, 1975); thus, Ca^{2+} appears to act as an internal messenger of light adaptation in these cells, possibly affecting some enzyme(s) important in the phototransduction cascade. Therefore, the regulation of $[Ca^{2+}]_i$ must be a critical feature of the signal processing function of the photoreceptor. In *Limulus,* $[Ca^{2+}]_i$ is influenced by light-activated release of Ca^{2+} from intracellular stores (Brown and Blinks, 1974), voltage-gated Ca^{2+} conductance in the plasma membrane (Lisman et al., 1982), Na^+/Ca^{2+} exchange in the plasma membrane (Lisman and Brown, 1972), and Ca^{2+} uptake into intracellular stores (Walz and Fein, 1983). Analogous to other neurons, there may be other factors that influence $[Ca^{2+}]_i$, for instance, soluble Ca^{2+}-binding proteins (Wood et al., 1980; Celio and Heizmann, 1981), active uptake of Ca^{2+} into intracellular organelles (Ebashi, 1980), or active extrusion of Ca^{2+} across the plasma membrane (Baker and DiPolo, 1984). The concerted action of these features (and any others of which we do not yet know) must impart to the cell a well regulated spatial and temporal profile of $[Ca^{2+}]_i$. Thus, Na^+/Ca^{2+} exchange is one among several processes that contribute to $[Ca^{2+}]_i$ regulation.

In the present study, we have examined temporal changes in $[Ca^{2+}]_i$ in response

to procedures designed to alter Na^+/Ca^{2+} exchange rates. We have also made estimates of $[Ca^{2+}]_i$ changes in response to illumination over a broad range of intensities. We provide evidence that Na^+/Ca^{2+} exchange can operate in forward or reverse directions. We have also measured changes in membrane currents that arise from procedures designed to alter Na^+/Ca^{2+} exchange and we provide evidence that Na^+/Ca^{2+} exchange in *Limulus* ventral photoreceptors is electrogenic.

Preliminary reports of some of this work have appeared previously in abstract form (Keller and O'Day, 1987; O'Day and Keller, 1987).

METHODS

Preparation

Limulus polyphemus were obtained from Marine Biological Labs, Woods Hole, MA. Ventral nerves were removed, desheathed, and treated with pronase (see Lisman et al., 1982 for details). Physiological measurements, microelectrode preparation, two-electrode voltage clamp, and pressure injection were similar to Lisman et al., (1982). During dissection and desheathing, the tissue was bathed in artificial seawater (ASW): 425 mM NaCl, 10 mM KCl, 22 mM $MgCl_2$, 26 mM $MgSO_4$, 10 mM $CaCl_2$, 15 mM Tris Cl, pH 7.8. Each experiment required salines with Na^+, Ca^{2+}, and Mg^{2+} concentrations that differed from those in ASW. "Normal" saline designates NaCl of 425 mM and $CaCl_2$ of 10 mM as in ASW; "0-Na^+" indicates that the NaCl was replaced mole for mole with LiCl, "0-Ca^{2+}" indicates that no $CaCl_2$ or EGTA was added to the solution and 10 mM additional $MgCl_2$ was added, "0-K^+" indicates that the KCl was replaced mole for mole with NaCl or LiCl. We measured that $[Ca^{2+}]$ of 0-Na, 0-Ca saline with a Ca^{2+}-selective electrode to be 4 μM.

Injection Solutions

The microelectrodes used for pressure injection were as follows: Na electrodes: 100 mM Na-aspartate, 100 mM K-aspartate, 10 mM HEPES, pH 7.0; aequorin electrodes: 10–12 mg/ml aequorin, 300 mM KCl, 10 mM HEPES, and 10 μM EGTA.

Optics

Experiments were performed using an inverted microscope with a planapochromat 20× objective lens (0.75 numerical aperture; 120379; Nikon, Inc., Garden City, NY). The beam through the objective was split so that infrared wavelengths were transmitted to an infrared-sensitive video camera for viewing the preparation, while visible wavelengths were reflected to a photomultiplier tube (8850 RCA, Lancaster, PA). We impaled cells under infrared illumination to maintain them in a relatively dark-adapted state, i.e., having low $[Ca^{2+}]_i$ (Brown and Blinks, 1974), so that little aequorin would be quenched before the experiment was underway. Fast, electronically controlled shutters were positioned in front of the video camera, the photomultiplier tube, and each light source to control the conditions of illumination. Illumination intensities shown in Figs. 2 and 8 are given in neutral density units (ND); each ND corresponds to a tenfold reduction in light intensity with 0 ND corresponding to the maximum available intensity, 1.0 mW/cm^2; white light was used (GE 1493 filament bulb). The output of the photomultiplier tube was sent to an electronic counter and photon counts as a function of time were stored digitally. The field of view of the photomultiplier tube was restricted by means of a window with which we could select a portion of the cell for viewing and measuring luminescence. The size of this window was approximately one-half the cross-

sectional area of a typical photoreceptor. The photomultiplier tube was cooled to achieve a low dark count rate of 1.6 ± 0.4 per s.

Aequorin

Aequorin was obtained from Dr. J. Blinks lab (Mayo Clinic, Rochester, MN). Aequorin electrodes were back-filled with aequorin injection solution, and the electrical connection between the solution and the measuring electronics was made with a platinum wire. Our aequorin electrode system was modeled after the design developed by Dr. Wesley Corson (Payne et al., 1986).

Aequorin luminesces in the presence of free Ca^{2+}; that is, on binding a calcium ion, an aequorin molecule emits a photon (Blinks et al., 1982). Detection of photons and analysis of aequorin luminescence in principle should enable us to make estimates of $[Ca^{2+}]_i$ (Allen and Blinks, 1978). We calibrated $[Ca^{2+}]$ vs. aequorin luminescence using the variable, $L_r = L(t)/L_{max}(t)$, where $L(t)$ is the number of photons emitted per second by aequorin at any time (t) and $L_{max}(t)$ is the peak number of photons that would be emitted per second if all the remaining aequorin available to luminesce at time t were suddenly exposed to a saturating concentration of Ca^{2+}. We have estimated L_{max} following the methods of Allen and Blinks (1978) by multiplying the total amount of light that could possibly be emitted by the available aequorin by the rate constant of aequorin consumption in the presence of a saturating concentration of Ca^{2+} (10/s at 21°C). The available aequorin in each cell at any given time was determined after the end of the experiment by keeping a continuous count of photons emitted vs. time and subtracting from the total photons counted during the experiment and afterwards when the cell was mechanically disrupted by an unfilled microelectrode.

There are several technical difficulties associated with measuring intracellular $[Ca^{2+}]$ with aequorin (Blinks et al., 1982); the uncertainty in stoichiometry of Ca^{2+}-aequorin binding, the uncertainty in the distribution of aequorin after it complexes with Ca^{2+} in localized regions, and the uncertainty about the intracellular ionic environment surrounding aequorin molecules all make it very difficult to quantify $[Ca^{2+}]_i$ levels. Our technique does not offer spatial resolution of $[Ca^{2+}]_i$ or of $[Ca^{2+}]_i$ changes. Spatial inhomogeneity may occur in the $[Ca^{2+}]_i$ changes associated with alterations of Na^+/Ca^{2+} exchange, similar to that in light-induced $[Ca^{2+}]_i$ increases (Harary and Brown, 1984; Levy and Fein, 1985; Payne and Fein, 1987). Our estimates of $[Ca^{2+}]_i$ may reflect a value weighted more toward the peak $[Ca^{2+}]_i$ than the average in the part of the cell sampled (Blinks et al., 1982). Further, local differences or changes in intracellular environments may create differences in aequorin-$[Ca^{2+}]_i$ calibration. For example, if changes in $[Li^+]_i$ or $[Na^+]_i$ accompany our manipulations of $[Li^+]_o$ and $[Na^+]_o$, intracellular aequorin luminescence may be affected slightly, since some univalent cations have small effects on aequorin luminescence in vitro (Moisescu and Ashley, 1977). In addition, since we have no independent measure of $[Ca^{2+}]_i$, our calibration of $[Ca^{2+}]_i$ vs. L_r is only approximate. Nonetheless, our approach allows us to estimate roughly the dark levels of $[Ca^{2+}]_i$, and it allows firm qualitative conclusions about the operation of Na^+/Ca^{2+} exchange in these photoreceptors.

The calibration curve shown in Fig. 1 is a recalculation of data from Blinks et al. (1982), incorporating estimates of the $[Mg^{2+}]_i$ and $[K^+]_i$ for *Limulus* ventral photoreceptors. Neither intracellular $[Mg^{2+}]$ nor intracellular $[K^+]$ has been reported for *Limulus* photoreceptors, and so we have used the values reported by Brown and Rydqvist (1982) in *Balanus* photoreceptors of 0.6 mM $[Mg^{2+}]_i$ and a $[K^+]_i$ value of 397 mM, which is similar to the $[K^+]_i$ found in squid axon (Hodgkin, 1951).

We injected aequorin with pressure pulses that ranged from 8 to 30 psi and 75 ms to 2 s in duration. The duration and strength of the pressure pulses were varied to meet the variability in the tendency of the microelectrodes to exude aequorin, as monitored optically (Corson

and Fein, 1983). Pulses were presented once every 3–10 s; and so the actual injection of aequorin occurred over a long period of time (10 min to >1 h). This strategy was used to allow the cell time to equilibrate osmotically after each injection and thereby to minimize the damage to the cell structure by injection of large volumes. Aequorin concentrations in the microelectrodes were near reported saturation level (Blinks et al., 1982).

We have examined whether the light emitted by aequorin in our experiments might be absorbed by the cell and induce significant Ca^{2+} release from intracellular stores. We rejected this possibility based on the following argument: At the peak relative aequorin luminescence in our experiments, we detected 35,000 photons/s; this translates to $\sim 5 \times 10^{-10}$ W/cm^2 (4.8 ND) at the photoreceptor rhabdomeral (photon absorbing) lobe for 1 s, assuming a photon energy of 5×10^{-19} J and a rhabdomeral cross section of 3×10^{-5} cm^2, and assuming that we detected 4% of the photons emitted (based on the geometry of our apparatus). O'Day et al. (1982) showed that *Limulus* ventral photoreceptors exhibit a very small decline from dark-adapted sensitivity with light of this intensity, suggesting that only a small light-induced rise in $[Ca^{2+}]_i$ would result. We have examined this problem more directly by measuring the relative luminescence induced by a 10-ms light flash of intensity tenfold greater than our estimates of maximum intensity of aequorin luminescence induced by the procedures in our experiments.

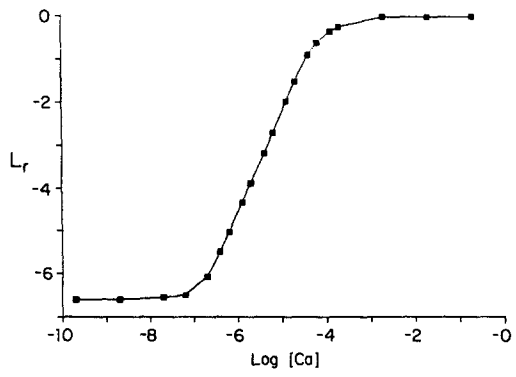

FIGURE 1. Aequorin calibration: plot of L_r, relative aequorin luminescence, vs. estimated $\log_{10}$ of molar $[Ca^{2+}]_i$ (abbreviated log [Ca]). The calibration curve used for making $[Ca^{2+}]_i$ estimates was calculated directly from published data (Blinks et al., 1982), detailing $[Mg^{2+}]$ dependence and $[K^+]$ dependence of Ca^{2+}-dependent aequorin luminescence. We have assumed $[Mg^{2+}]_i$ = 0.6 mM and $[K^+]_i$ = 397 mM in generating this curve.

We presented test flashes (10 ms) of 3.8 ND to a cell filled to approximately the same aequorin concentration as that in Figs. 3 and 4, below. Fig. 2 shows that the resulting L_r peaked at ~2.25 log units below the maximum measured L_r induced during the procedures in Figs. 3 and 4. Therefore, we consider it very unlikely that aequorin luminescence initiated substantial Ca^{2+} release in any of our experiments. In the worst case, aequorin may initiate ~2 μM of Ca^{2+} release near the peak of a 21 μM $[Ca^{2+}]_i$ rise induced by Na^+ removal.

RESULTS

The presence of a Na^+/Ca^{2+} exchange process in the photoreceptor membrane would have specific predictable consequences for the regulation of $[Ca^{2+}]_i$. Our strategy in this study was first to determine whether we could estimate levels of $[Ca^{2+}]_i$ in an unilluminated photoreceptor, and then to detect predictable changes in $[Ca^{2+}]_i$ during procedures designed to affect the rate of Na^+/Ca^{2+} exchange. We have used three such procedures: (*a*) changing extracellular $[Na^+]$, (*b*) changing extracellular $[Ca^{2+}]$, (*c*) changing intracellular $[Na^+]$.

Dark Levels of $[Ca^{2+}]_i$

We asked first whether we could detect resting levels of $[Ca^{2+}]_i$ in an unilluminated, dark-adapted cell, where $[Ca^{2+}]_i$ is expected to be quite low (Lisman and Brown, 1972). To determine $[Ca^{2+}]_i$, we compared the total number of photons detected in a 5-min period before aequorin injection with that detected afterwards. In most cells (35 of 60) there was no significant difference between luminescence measured before and that measured after aequorin injection. This suggested that resting $[Ca^{2+}]_i$ was sufficiently low and the amount of injected aequorin was sufficiently small to preclude detection of a resting aequorin luminescence. In several of these cells, we measured the luminescence resulting from a bright light flash to determine whether $[Ca^{2+}]_i$ could be raised to a detectable level by illumination (Brown and Blinks, 1974). We found that in 5 of 5 cells a 1-ND, 30-ms flash could elicit a detectable aequorin luminescence signal and that later injection of more aequorin could increase the flash-induced luminescence signal. In all of these cells, however, we were unable to detect a difference in resting dark luminescence between the measurements made before and those made after aequorin injections.

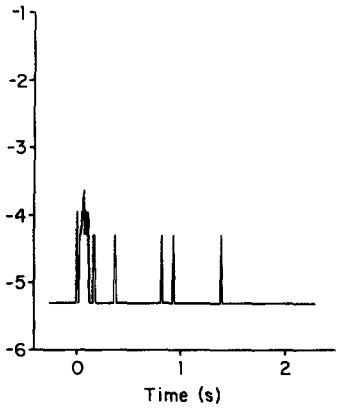

FIGURE 2. L_r rise induced by dim lights was small compared with L_r rises induced by solution changes. A dim flash (3.8 ND, 10 ms) was presented at $t = 0$ to a cell filled to approximately the same aequorin concentration as that in Fig. 3. Luminescence data were collected in 10-ms bins.

In other cells (25 of 60), we did observe a significant difference in resting dark luminescence before and after aequorin injection. These cells fell into two categories: (*a*) 8 of 25 cells had relatively small light-induced luminescence changes yet exhibited large luminescence changes in response to the removal of extracellular Ca^{2+}. In these cases, estimated $[Ca]_i$ was high at rest (2.1 ± 0.7 μM; SD). (*b*) 17 of 25 cells exhibited relatively large light-induced luminescence changes and small luminescence changes in response to removal of extracellular Ca^{2+}. In these cells, estimated $[Ca^{2+}]_i$ was low at rest (0.66 ± 0.09 μM; SD). These two classes of observations suggested that in the former group the cells may have been damaged by the impaling aequorin electrode, permitting easy diffusion of Ca^{2+} into the cell, while the cells in the latter group were less seriously affected.

We therefore examined the idea that an impaling electrode could disrupt the membrane integrity and result in a large rise in $[Ca^{2+}]_i$. We preinjected a photoreceptor with aequorin and monitored luminescence at regular intervals after removal of the aequorin electrode. We then impaled the cell with a second electrode and

monitored membrane potential. We found in 6 of 12 cells that, after the second impalement, the resting luminescence rose to a peak and then declined to a steady level that was higher than that preceding the second impalement ($L' = L_{before}/L_{after} = 2.7 \pm 1.2$; SD). In the remaining cells there was no effect of the second impalement on the resting luminescence ($L' = 1.0 \pm 0.2$; SD). This suggests that impalement alone can be sufficient to raise $[Ca^{2+}]_i$ significantly.

The estimated $[Ca^{2+}]_i$ from all cells from which we could detect a change in luminescence after aequorin injection (category a) was 1.1 μM ± 0.8 μM (SD); the estimated $[Ca^{2+}]_i$ from all cells we judged to be minimally affected by impalement (category b) was 660 nM ± 70 nM (SD). In no experiment were we able to detect a resting luminescence in 0-Ca^{2+} saline ($L_{before}/L_{after} = 0.99 \pm 0.11$; SD). All the cells reported in the following sections were judged to be minimally affected by impalement (category b).

Effects of Changing Extracellular $[Na^+]$ on $[Ca^{2+}]_i$

If a Na^+/Ca^{2+} exchange process in the plasma membrane keeps $[Ca^{2+}]_i$ low by constantly extruding Ca^{2+}, then blocking forward exchange (Na_o^+-dependent Ca^{2+} extrusion) and stimulating reverse exchange should cause $[Ca^{2+}]_i$ to rise, and restoring forward exchange should cause $[Ca^{2+}]_i$ to fall. To examine these predictions in the *Limulus* ventral photoreceptor, we removed and then restored Na_o^+ while monitoring changes in $[Ca^{2+}]_i$ using aequorin, previously injected into the cells. Removal of Na_o^+ should block forward exchange and stimulate reverse exchange (DiPolo, 1973). Fig. 3 *A* illustrates the results of removing Na^+ from the saline bathing these cells. Na_o^+ removal causes a fast rise in luminescence, indicating a rise in $[Ca^{2+}]_i$. Luminescence reached a peak and then declined, approaching a "plateau" level in its rate of decline. No rise in $[Ca^{2+}]_i$ was detected when Na_o^+ was removed from 0-Ca^{2+} saline (trace *3*). This suggests that the origin of the Ca_i^{2+} in traces *1* and *2* was the extracellular saline. These observations are thus consistent with the interpretation that Na^+ removal caused Ca^{2+} to enter the cytosol from the bathing medium by favoring reverse Na^+/Ca^{2+} exchange. The decline from peak luminescence in Fig. 3 *A* indicates that, after Ca^{2+} entry, a substantial amount of Ca^{2+} was somehow removed from the cytosol (and/or that [aequorin] was locally reduced). When Na^+ was restored to the bathing saline, the luminescence quickly fell to baseline, indicating a fast decline in $[Ca^{2+}]_i$, which is consistent with the interpretation that Na_o^+ restoration very quickly restored Ca^{2+} extrusion by forward Na^+/Ca^{2+} exchange. Na^+ removal caused a small depolarization (8 mV in presentation 1, Fig. 3 *A*; 5 mV in presentation 2). In other cells, Na^+ removal caused large changes in $[Ca^{2+}]_i$ with no apparent changes in membrane potential. In 0-Ca^{2+} saline, a biphasic change in membrane potential was observed when Na^+ was removed (presentation 3).

To determine the time courses of $[Ca^{2+}]_i$ changes, we plotted the estimated $[Ca^{2+}]_i$ vs. time (Fig. 3 *B*). This figure illustrates that when the cell was bathed in normal saline, the estimated resting $[Ca^{2+}]_i$ was in the neighborhood of 0.5–0.6 μM. $[Na^+]_o$ removal caused $[Ca^{2+}]_i$ to rise rapidly to a peak of ~21 μM. The decline from the peak was rapid at first, and then it slowed, with $[Ca^{2+}]_i$ reaching roughly 5.5 μM

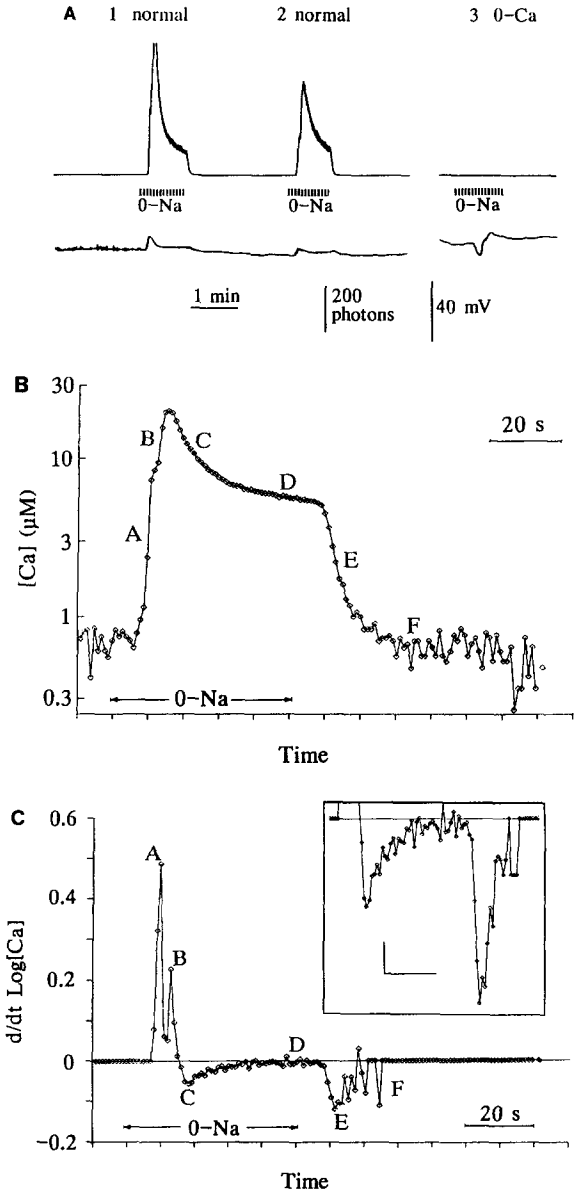

FIGURE 3. Na^+ removal: normal Ca^{2+}, 0-K^+ saline (experiment 1). Removal of Na_o^+ caused a rise in $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}. (*A*) The results of three separate presentations of 0-Na saline are shown. Presentations 1 and 2 were made after 15 min in normal 0-K^+saline; presentation 3 was made in 0-Ca^{2+}, 0-K^+ saline. Top trace, photons counted; bottom trace, membrane potential. Na^+-removal caused large increases in $[Ca^{2+}]_i$ in normal saline, but no rise in $[Ca^{2+}]_i$ was detected in 0-Ca^{2+} saline (after 5 min of 0-Ca^{2+} exposure). Luminescence data are direct analog output of photon counter filtered at 10 Hz. Membrane potential changes were measured with the aequorin-filled microelectrode. Na^+ removal caused a small transient depolarization of membrane potential; Na^+ restoration caused a small repolarization (1 and 2). In the absence of Ca_o^{2+} (3), Na^+ removal caused a small transient hyperpolarization followed by a small transient depolarization. (*B*) Digitized data from presentation 1 (*A1*) were collected and summed in 1-s bins. The estimated $[Ca^{2+}]_i$ calculated from the calibration curve of Fig. 1 is plotted vs. time. Six phases are labeled in the figure: *A*, fast rise in $[Ca^{2+}]_i$ after Na^+ removal; *B*, slow rise in $[Ca^{2+}]_i$; *C*, Initial decline from peak $[Ca^{2+}]_i$; *D*, slower decline in $[Ca^{2+}]_i$ approaching plateau; *E*, initial phase of $[Ca^{2+}]_i$ decline after restoration of Na^+; *F*, final decline of $[Ca^{2+}]_i$ to baseline. (*C*) The rate of change in estimated $\log_{10}$ molar $[Ca^{2+}]_i$ is plotted vs. time to illustrate the rates of $[Ca^{2+}]_i$ change during the various phases described in Fig. 3 *B*. The inset clarifies the regions below $d/dt(\log [Ca^{2+}]_i) = 0$. The ordinate is the change in $\log_{10}$ molar $[Ca^{2+}]_i$ per 1-s interval. The calibration bars in the inset represent 0.02 and 20 s. The data were filtered in the following way to reduce the noise in the figure: For values of $[Ca^{2+}]_i$ above 1.75 μM, the value plotted is the actual change in $\log_{10} \cdot [Ca^{2+}]_i$ per second interval; for $[Ca^{2+}]_i < 1.75$ but >1 μM the values plotted are the average over a 3-s (2-s for inset) interval; for values <1 μM the value 0 was plotted.

before $[Na^+]_o$ was restored. Na^+ restoration then caused $[Ca^{2+}]_i$ to fall back to baseline. Fig. 3 *C* illustrates directly the rate of change in estimated $[Ca^{2+}]_i$ vs. time.

The changes in $[Ca^{2+}]_i$ in Fig. 3 had several distinct phases that are labeled in Fig. 3, *B* and *C* for clarity. There were two distinct components of the quick rise in $[Ca^{2+}]_i$: a fast early rise (phase *A*) of ~1 log unit/2 s, interrupted by a "glitch" during which $[Ca^{2+}]_i$ appears to have actually declined (compare Fig. 3 *A*), followed by a second fast rise (phase *B*) of ~1 log unit/5 s to a peak. The rate of fall from peak estimated $[Ca^{2+}]_i$ (phase *C*) was initially 1 log unit in ~20 s (from 20 to 8 μM) and this rate declined (phase *D*) to 1 log unit in ~280 s (from 7.5 to 5.5 μM). The inset of Fig. 3 *C* shows the rates of $[Ca^{2+}]_i$ decline on an expanded scale. Restoration of Na_o^+ caused a substantial increase in the rate of $[Ca^{2+}]_i$ decline (phase *E*) to 1 log unit in ~9 s (4–1.2 μM), and this rate gradually declined (phase *F*) to 1 log unit in ~45 s down to baseline (1–0.56 μM). Similar results were found in six of six cells studied in this way.

Effects of Changing Extracellular $[Ca^{2+}]$

If there is a Na^+/Ca^{2+} exchange process in the plasma membrane, we would expect stimulation of reverse exchange in the absence of forward exchange to cause a significant rise in $[Ca^{2+}]_i$. Thus, if both forward and reverse exchange are inhibited by the removal of Na_o^+ and Ca_o^{2+}, and if Ca_o^{2+} is subsequently restored, we expect an increase in $[Ca^{2+}]_i$ due to reverse exchange (assuming $[Na^+]_i$ does not drop substantially). On the other hand, in 0-Ca^{2+}, normal-Na^+ saline, the addition of Ca_o^{2+} should not cause such a large rise in $[Ca^{2+}]_i$ since Ca^{2+} extrusion would be ongoing in the presence of Na_o^+.

To examine these predictions, we bathed the preparation of 0-Na^+, 0-Ca^{2+} saline and then restored 10 mM Ca^{2+} to the saline (Fig. 4, same photoreceptor as in Fig. 3). When the preparation was bathed in 0-Na^+, 0-Ca^{2+} saline, estimated $[Ca^{2+}]_i$ was 0.36 μM, a value indistinguishable from background. When Ca^{2+} was reintroduced to the saline, $[Ca^{2+}]_i$ rose quickly to a peak of ~16.5 μM, and declined to a plateau of ~5.3 μM. Luminescence and membrane potential vs. time are shown in Fig. 4 *A*. The cell hyperpolarized after Ca^{2+} addition in presentation 1, but in presentation 2 there was a multiphasic response; in 0-Ca^{2+} saline, Ca^{2+} addition caused a multiphasic change in membrane potential as well. Estimates of $[Ca^{2+}]_i$ are plotted vs. time in Fig. 4 *B*. The rates of change in estimated $[Ca^{2+}]_i$ are plotted vs. time in Fig. 4 *C*, reaching 1 log unit rise in ~2 s (phase *A*). The later rise (phase *B*) was ~1 log unit in 8 s. The rate of decline from peak $[Ca^{2+}]_i$ (phase *C*) was initially ~1 log unit in 25 s and then slowed to ~1 log unit in 300 s (phase *D*). When $[Ca^{2+}]_o$ was subsequently reduced back to 0-Ca^{2+} saline levels, the $[Ca^{2+}]_i$ decreased at a slow rate (1 log unit in 110 s, phase *E'*). Subsequent addition of Na_o^+ caused a rapid decline in $[Ca^{2+}]_i$ back to baseline (phase *F'*). Similar results were obtained in six of six cells.

It is instructive to compare the rates of $[Ca^{2+}]_i$ changes in the second experiment (Fig. 4) with those of the first (Fig. 3). Fig. 5, *A* and *B* illustrates that the time courses of the rise of $[Ca^{2+}]_i$ were remarkably similar in the two experiments, as were those of the decline of $[Ca^{2+}]_i$. The time courses of both the transient rise and the decline were remarkably similar in each trial in this cell ($n = 5$). This phenomenon was observed in six of six cells studied in this way.

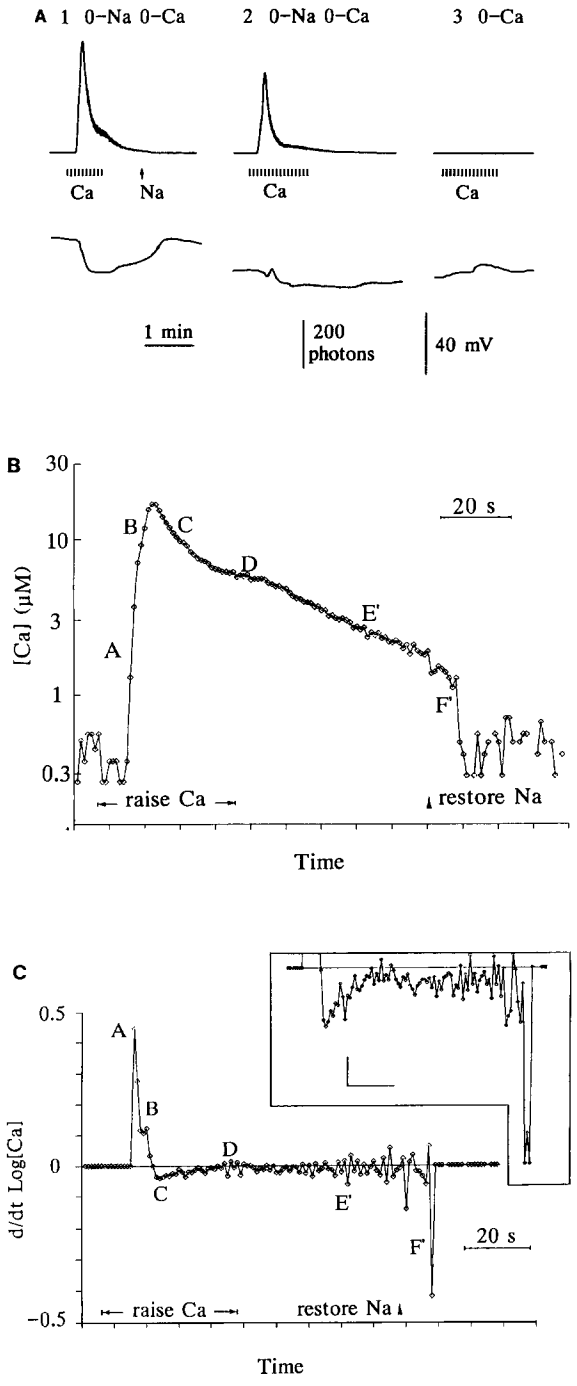

FIGURE 4. Na^+ removal: 0-Ca^{2+}, 0-K^+ saline (experiment 2). Raising $[Ca^{2+}]_o$ caused a large rise in $[Ca^{2+}]_i$ in the absence of Na_o^+. (*A*) The results of two separate presentations of 10 mM Ca^{2+} in 0-Na^+ saline are shown. The cell was bathed initially in 0-Na^+, 0-Ca^{2+}, O-K^+ saline for 10 min. The top trace indicates photons counted, the bottom trace indicates membrane potential. Raising $[Ca^{2+}]_o$ to normal levels (10 mM) caused a large transient increase of estimated $[Ca^{2+}]_i$ when Na^+ was absent from the saline. No large rise in $[Ca^{2+}]_i$ was observed in the presence of normal $[Na^+]_o$ (presentation 3). The data were collected from the same cell as in Fig. 3. Ca^{2+} addition in 0-Na^+ saline caused a significant hyperpolarization in presentation 1 and a small, biphasic potential change in presentation 2. A slow repolarization was observed when Ca^{2+} was again removed. (*B*) As in Fig. 3, the estimated $[Ca^{2+}]_i$ is plotted vs. time. Six phases of $[Ca^{2+}]_i$ change are evident: *A*, fast rise in $[Ca^{2+}]_i$ after Ca^{2+} addition to saline; *B*, slowed rise in $[Ca^{2+}]_i$; *C*, initial decline from peak $[Ca^{2+}]_i$; *D*, slower decline in $[Ca^{2+}]_i$ approaching plateau; *E'*, $[Ca^{2+}]_i$ decline after Ca^{2+} removal from saline; *F'*, fast $[Ca^{2+}]_i$ decline to baseline after restoration of Na^+ to saline. (*C*) The rate of change in estimated $\log_{10}$ molar $[Ca^{2+}]_i$ from *A* is plotted vs. time, and filtered as in Fig. 3 *C*. Calibration bars of inset represent 0.02 and 20 s.

Fig. 5 *B* allows us to compare the rate of $[Ca^{2+}]_i$ decline in the presence of Na_o^+ with that in its absence. $[Ca^{2+}]_i$ declined quickly in experiment 1 when normal $[Na^+]_o$ was restored to the saline. In experiment 2, $[Ca^{2+}]_i$ declined slowly when Ca^{2+} was removed from the saline and then declined quickly to baseline when Na^+ was restored. These observations provide clear evidence for a fast Na_o^+-dependent $[Ca^{2+}]_i$ decline.

We tested the prediction made above that the $[Ca^{2+}]_i$ rise induced by raising $[Ca^{2+}]_o$ should not be as great in the presence of Na_o^+ as in its absence. We restored normal $[Ca^{2+}]_o$ to aequorin-filled cells bathed in 0-Ca^{2+}, normal Na^+ saline. A significant difference in the relative luminescence upon addition of Ca_o^{2+} was apparent in only three of six cells studied (e.g., Fig. 6). But in those three cells the rise in

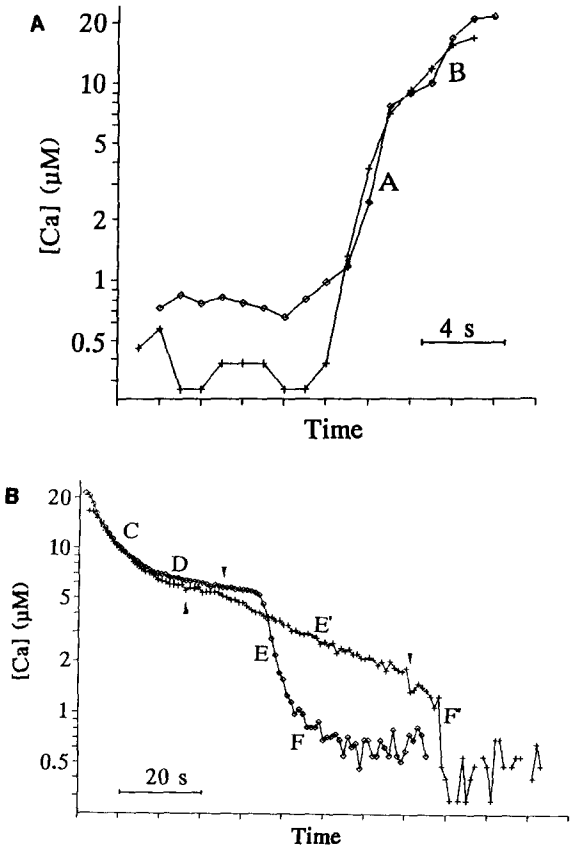

FIGURE 5. Comparison of the rates of $[Ca^{2+}]_i$ change. Estimated $[Ca^{2+}]_i$ from Fig. 3 *A* (experiment 1, ◊) and Fig. 4 *A* (experiment 2, +) are plotted together vs. time to compare and contrast the time courses of $[Ca^{2+}]_i$ change. (*A*) Phases *A* and *B*: The time courses of estimated $[Ca^{2+}]_i$ rise were remarkably similar at similar $[Ca^{2+}]_i$ levels in the two experiments even though the initial values of $[Ca^{2+}]_i$ were different. (*B*) Phases *E* and *F*: The time course of estimated $[Ca^{2+}]_i$ decline differed between the two experiments. Na^+ restoration caused a fast decline of $[Ca^{2+}]_i$ in experiment 1 (phase *E*); however, Ca^{2+} removal caused a much slower decline of $[Ca^{2+}]_i$ in experiment 2 (phase *E'*). Subsequent restoration of Na^+ in experiment 2 led to a fast decline in $[Ca^{2+}]_i$ (phase F').

$[Ca^{2+}]_i$, was larger in the absence of Na_o^+ than in its presence. The most marked rise in $[Ca^{2+}]_i$ in these experiments was ~1.9 μM; this compares with a rise in $[Ca^{2+}]_i$ in the absence of Na_o^+ of roughly 20 μM (Fig. 4).

Effects of Changing Intracellular [Na^+]

If Na^+/Ca^{2+} exchange is indeed reversible, we would expect that in the absence of forward exchange an additional rise in $[Ca^{2+}]_i$ might be observable when reverse

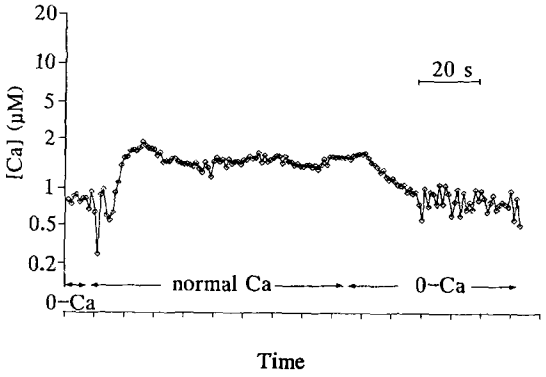

FIGURE 6. Ca^{2+} addition to normal Na^+ saline. Raising $[Ca^{2+}]_o$ caused a small rise in $[Ca^{2+}]_i$ in the presence of extracellular Na^+. Estimated $[Ca^{2+}]_i$ is plotted vs. time in a different cell from that in Figs. 3–5. In this case, the cell was bathed initially in 0-Ca^{2+} normal Na^+ saline. When Ca^{2+} was restored, $[Ca^{2+}]_i$ rose significantly; when 0-Ca^{2+} saline was subsequently reintroduced, $[Ca^{2+}]_i$ slowly declined. In three of six cells we found a significant difference between normal Ca^{2+} and 0-Ca^{2+} conditions: the average $[Ca^{2+}]_i$ in normal Ca^{2+} saline was 1.26 ± 0.40 μM and in 0-Ca^{2+} saline it was 0.55 ± 0.18 μM. In three cells we found no significant difference. In these cells, $[Ca^{2+}]_i$ averaged 0.51 ± 0.13 μM in normal Ca^{2+} saline and 0.50 ± 0.11 μM in 0-Ca^{2+} saline.

exchange was enhanced by increasing $[Na^+]_i$. To examine this prediction, we injected Na^+ into the cell and monitored changes in $[Ca^{2+}]_i$. The cell was bathed initially in 0-Na^+ saline for 5 min while the $[Ca^{2+}]_i$ level stabilized (estimated $[Ca^{2+}]_i$ = 2.4 μM). A series of 100-ms pressure pulses of Na^+ injection solution was then presented, during which time $[Ca^{2+}]_i$ began to rise (Fig. 7). Estimated $[Ca^{2+}]_i$ peaked at 5.3 μM. We repeated the experiment in 0-Ca^{2+} saline, and found no detectable rise in $[Ca^{2+}]_i$. These results provide evidence for Na_i^+-stimulated Ca^{2+} entry, and they suggest that the Ca^{2+} entry in the absence of Na_o^+ (Figs. 3–5) may not be simply a manifestation of a reduction of Ca^{2+} extrusion.

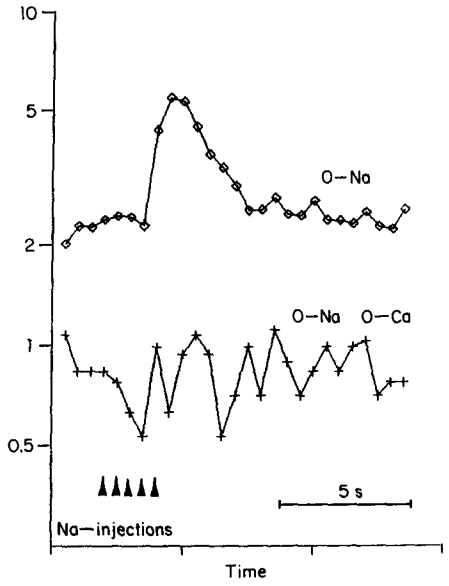

FIGURE 7. Na^+ injection in 0-Na^+ normal Ca^{2+} saline. Na^+ injection caused a rise in $[Ca^{2+}]_i$ in 0-Na^+ saline in the presence of Ca_o^{2+}. A cell was impaled with a single microelectrode filled with Na injection solution. At each arrow in the figure, Na^+ was injected by a 100-ms pressure pulse applied to the back of the electrode at intervals of 1 s. In the upper trace, a $[Ca^{2+}]_i$ rise was observed in 0-Na^+, normal Ca^{2+} saline. No rise in $[Ca^{2+}]_i$ was observed in 0-Na^+, 0-Ca^{2+} saline.

Comparison with Light-induced Increases in $[Ca^{2+}]_i$

Because Na^+/Ca^{2+} exchange probably plays a physiological role during the light response (P. M. O'Day, M. P. Gray-Keller, and M. Lonergan, manuscript in preparation), it is of interest to compare the increase in $[Ca^{2+}]_i$ induced by our procedures for stimulating reverse exchange with the rise in $[Ca^{2+}]_i$ induced by illumination. We presented a series of test flashes (50 ms of white light) ranging in intensity from −6 to 0 ND, and monitored changes in aequorin luminescence after each flash in normal saline. Between each flash the cell was allowed to dark-adapt to a

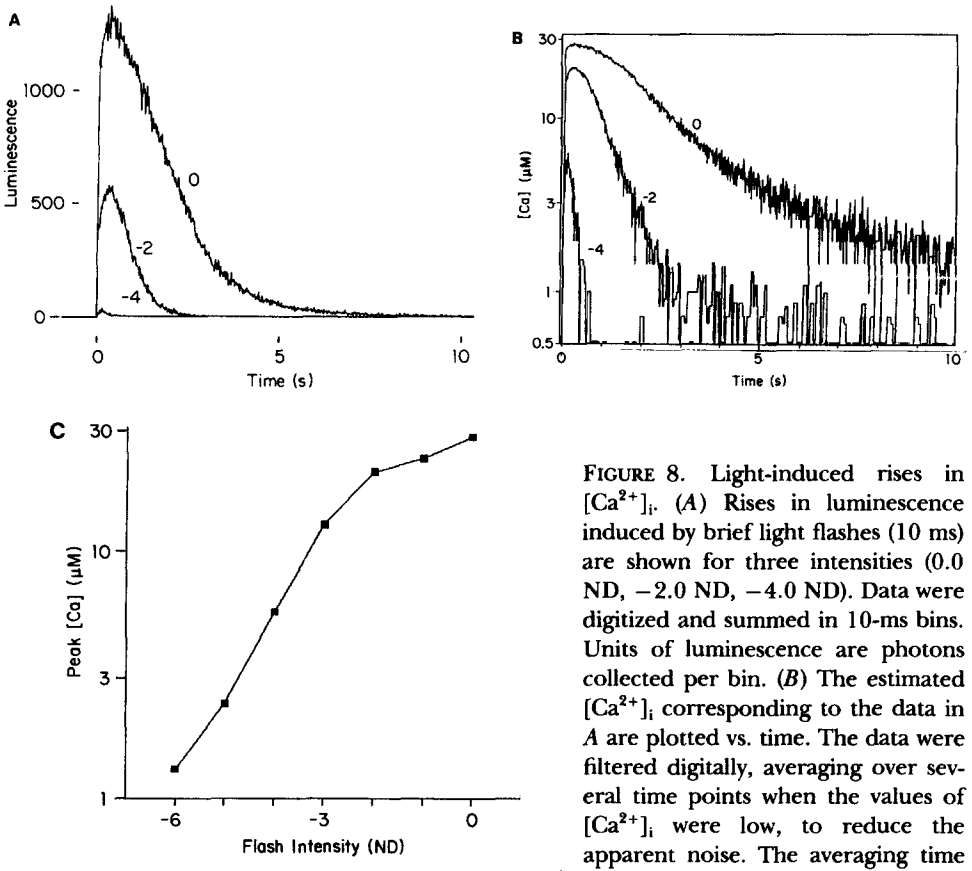

FIGURE 8. Light-induced rises in $[Ca^{2+}]_i$. (*A*) Rises in luminescence induced by brief light flashes (10 ms) are shown for three intensities (0.0 ND, −2.0 ND, −4.0 ND). Data were digitized and summed in 10-ms bins. Units of luminescence are photons collected per bin. (*B*) The estimated $[Ca^{2+}]_i$ corresponding to the data in *A* are plotted vs. time. The data were filtered digitally, averaging over several time points when the values of $[Ca^{2+}]_i$ were low, to reduce the apparent noise. The averaging time was calculated with the equation: $t = 10 \text{ ms} * \text{Int}\,[10^{\wedge}(-1.077 * L_r - 0.388)]$. (*C*) The estimated peak light-induced rise in $[Ca^{2+}]_i$ is plotted vs. flash intensity for the experiment described in *A*.

criterion level (threshold intensity = −6 ND of 530 nm light for 1 mV light response). Results for three different test flash intensities are shown in Fig. 8. The results in Fig. 8 *A* indicate that in each case the $[Ca^{2+}]_i$ rose quickly and then quickly declined. The time course of $\log_{10}[Ca^{2+}]_i$ change is more clearly seen in Fig. 8 *B*, in which the estimated $\log_{10}[Ca^{2+}]_i$ vs. time after the light flash is plotted. In Fig. 8 *C*, we have plotted the peak values of estimated $[Ca^{2+}]_i$ as a function of the intensity of

the test flash that evoked the $[Ca^{2+}]_i$ rise. Log_{10} $[Ca^{2+}]_i$ increased nearly linearly with the logarithm of intensity up to $I = -2$ ND ($[Ca^{2+}]_i = 21$ μM). At intensities above -2 ND, the curve began to level off. This region is near the region where saturation begins in the aequorin-Ca^{2+} calibration curve (Fig. 1). This curve may thus reflect either aequorin-Ca^{2+} saturation or saturation of light-induced Ca^{2+} release at high intensities.

From Fig. 8 it is clear that light-induced $[Ca^{2+}]_i$ rises can be significantly greater than $[Ca^{2+}]_i$ rises we have induced by Na_o^+ removal. Peak $[Ca^{2+}]_i$ reached an estimated 30 μM after the flash of (0 ND) white light, compared with 21 μM induced by Na_o^+ removal (Fig. 3).

Membrane Currents

Na^+/Ca^{2+} exchange in several other tissues has been shown to be electrogenic (e.g., Blaustein, 1984), suggesting possible molecular mechanisms underlying Na^+/Ca^{2+} exchange. To determine whether Na^+/Ca^{2+} exchange might be electrogenic in

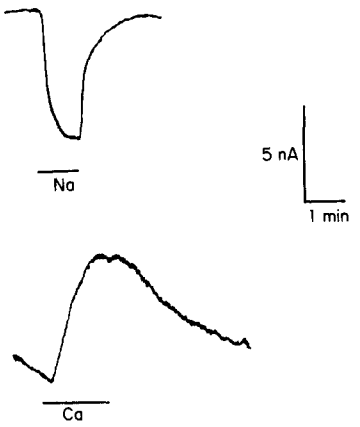

FIGURE 9. Na_o^+-induced inward current and Ca_o^{2+}-induced outward current. The photoreceptor, under constant voltage clamp to -80 mV, was bathed in 0-K^+, 0-Na^+, 0-Ca^{2+} saline in the dark. K^+ was removed to inhibit electrogenic Na^+/K^+-coupled transport (Brown and Lisman, 1972). First trace, inward current resulted from Na_o^+ addition (425 mM). Second trace, outward current resulted in Ca_o^{2+} addition (10 mM).

Limulus ventral photoreceptors, we measured changes in membrane currents induced by changes in $[Na^+]_o$ or $[Ca^{2+}]_o$ under voltage clamp. Cells were bathed in 0-Na^+, 0-Ca^{2+} saline and voltage clamped to -80 mV to eliminate currents arising from voltage-activated conductances (Lisman et al., 1982). We eliminated K^+ from the bath to prevent currents arising from the electrogenic Na^+/K^+-exchange pump (Brown and Lisman, 1972), and we kept the cells in the dark to avoid light-activated currents (Millecchia and Mauro, 1969*b*). Fig. 9 shows that the addition of $[Na^+]_o$ induced a slowly developing inward current of ~10 nA. The addition of Ca_o^{2+}, in contrast, induced a slowly developing outward current of ~7 nA. Response sizes were quite variable in both cases: Na_o^+-induced inward currents averaged 7 ± 6 nA (SD); Ca_o^{2+}-induced outward currents averaged 5 ± 4 nA (SD). In two of six trials Ca_o^{2+}-induced outward currents were blocked by 100 μM $LaCl_3$. In six trials, 100 μM $LaCl_3$ did not block the Na_o^+-induced inward currents. These results are consistent with the idea that Na^+/Ca^{2+} exchange in *Limulus* ventral photoreceptor is electrogenic.

DISCUSSION

We have found in *Limulus* ventral photoreceptors that Na_o^+ removal caused a rise in $[Ca^{2+}]_i$ in normal Ca^{2+} saline, but not in 0-Ca^{2+} saline; Na_o^+ restoration subsequently caused a fast decline to baseline $[Ca^{2+}]_i$. Similarly, the addition of Ca_o^{2+} to cells bathed in 0-Na^+, 0-Ca^{2+} saline caused a large rise in $[Ca^{2+}]_i$ with a nearly identical time course; a return to 0-Na^+, 0-Ca^{2+} saline caused a very slow decline in $[Ca^{2+}]_i$, but subsequent addition of Na_o^+ caused a fast decline to baseline $[Ca^{2+}]_i$. Intracellular injection of Na^+ in the absence of Na_o^+ caused a large rise in $[Ca^{2+}]_i$ in normal Ca^{2+} saline, but not in 0-Ca^{2+} saline. Further, we have observed inward currents resulting from raising extracellular $[Na^+]$ and outward currents resulting from raising extracellular $[Ca^{2+}]$ in 0-Na^+, 0-Ca^{2+} saline. These qualitative results provide strong evidence for an electrogenic Na^+/Ca^{2+} exchange process in the plasma membrane of *Limulus* ventral photoreceptors that is capable of operating in forward or reverse directions. The data suggest that this process can extrude Ca^{2+} very quickly and that it can create large transmembrane $[Ca^{2+}]$ gradients by reducing $[Ca^{2+}]_i$ to very low levels.

Electrogenicity

Measurements of currents induced by changes in $[Ca^{2+}]_o$ or $[Na^+]_o$ suggest that Na^+/Ca^{2+} exchange in *Limulus* ventral photoreceptors may be electrogenic. A brief calculation indicates that if the currents shown in Fig. 9 arose principally from electrogenic exchange, Ca^{2+} must be very highly buffered intracellularly. The outward current has a peak value of 7 nA. Assuming that this arises entirely from a Na^+/Ca^{2+} exchange process with a 3:1 stoichiometry, this suggests that 70 fmol of Ca^{2+} enter the cell per second. But the net rise in $[Ca^{2+}]_i$ under similar conditions (Fig. 3) was only 0.25 fmol/s (assuming a 0.5 μM/s rise in $[Ca^{2+}]_i$ and a cell volume of 5×10^{-10} liter). Thus, in this case the Ca^{2+} buffers and Ca^{2+}-sequestering elements must remove ~99.65% of the entering free Ca^{2+}. This is similar to the report of McNaughton et al. (1986) that only 5% of Ca^{2+} entering the cell after Na_o^+ removal from salamander rod photoreceptors appeared as free Ca^{2+}.

The outward currents we have observed (Fig. 9) in response to Ca^{2+} addition may be masked by an inward current induced by a rise in $[Ca^{2+}]_i$ caused by reverse exchange. Payne et al. (1986) have shown that artificially raising $[Ca^{2+}]_i$ evokes an inward current that may arise from the activation of the light-dependent conductance.

The changes in membrane potential that accompany changes in $[Na^+]_o$ or $[Ca^{2+}]_o$ vary from cell and from trial to trial or the same cell (not shown). We have observed depolarization due to Na^+ removal (e.g., Fig. 3) in many cells, but other cells showed no observable change in membrane potential. The origins of the depolarization in Fig. 3 and those of the hyperpolarization in Fig. 4 that accompany the rise in $[Ca^{2+}]_i$ are not known. If an electrogenic Na^+/Ca^{2+} exchange process were the only process contributing to a change in membrane potential, a hyperpolarization would accompany the rise in $[Ca^{2+}]_i$; and so it is clear that the change in membrane potential in Fig. 3 *A* is not due solely to electrogenic Na^+/Ca^{2+} exchange. It is possible that a component of the depolarization induced by Na^+ removal in Fig. 3 *A* arose from the

opening of light-dependent channels induced by the rise in $[Ca^{2+}]_i$, similar to the depolarization observed after Ca^{2+} injection (Payne et al., 1986).

Resting $[Ca^{2+}]_i$ and Light-induced $[Ca^{2+}]_i$ Rises

The results shown in Figs. 5 and 6 suggest that a resting level of $[Ca^{2+}]_i$ in normal seawater can be estimated. The values we present are likely to be overestimates (see Methods). In normal $[Na^+]_o$ saline, L_r was clearly not below the limit of detection because lower L_r values were measured for 0-Ca^{2+} saline. Our ability to detect a difference between these two conditions should depend on the amount of intracellular aequorin and the magnitude of the difference in $[Ca^{2+}]_i$ in the two conditions. It is possible that at least part of the difference shown in Fig. 6 arose from Ca^{2+} leakage induced by impalement with the aequorin-filled electrode; however, the fact that this cell appeared to be quite light sensitive before and after each aequorin injection (not shown) suggests that Ca^{2+} leakage was not sufficient to impair substantially the health of the cell. Light sensitivity was judged in this case by the level of quantum bump, or discrete wave, activity (Dodge et al., 1968). The fact that we could estimate resting $[Ca^{2+}]_i$ in some cells and not in others is not surprising. Differences in resting $[Ca^{2+}]_i$ might be expected from cell to cell, since light sensitivity can vary greatly in a cell at rest (Lisman and Brown, 1975), and since light sensitivity is related to $[Ca^{2+}]_i$ (Lisman and Brown, 1972; Fein and Charlton, 1977; Nagy and Stieve, 1983; Levy and Fein, 1985). Furthermore, Ca^{2+} leakage induced by membrane disruption by the impaling microelectrode may be significant in some cells and not others, depending on the quality of the impalement.

The peak estimated light-induced $[Ca^{2+}]_i$ rise in our experiments is lower than that estimated by Brown et al., (1977) (at least 100 μM) using arsenazo III. Our estimates of $[Ca^{2+}]_i$ in normal saline under resting conditions also differ somewhat from those of Levy and Fine (1985), who reported a mean resting $[Ca^{2+}]_i$ of 3.5 μM using Ca^{2+}-selective microelectrodes. We find a value of ~0.6 μM, about a factor of six lower. On the other hand, when the cells were bathed in nominally Ca^{2+}-free saline, we found a value of 0.36 μM or less (below our detection limit), which is in rough agreement with the values Levy and Fein (1985) observed in three out of six cells (their Table VI). The time-dependent changes in $[Ca^{2+}]_i$ induced by illumination that we estimate, are similar to those reported by Levy and Fein (1985). They reported a peak rise in $[Ca^{2+}]_i$ of ~44 μM in response to a bright flash, and their data indicate that the decline to half-maximal $[Ca^{2+}]_i$ occurred in ~1.5 s. We found a 30 μM rise in $[Ca^{2+}]_i$ in response to a bright flash of similar intensity followed by a decline to half-maximal $[Ca^{2+}]_i$ in ~1.5 s. However, our measurements near the peak light-induced rise in $[Ca^{2+}]_i$ approach the saturation of our aequorin calibration curve (Fig. 1), and so the accuracy of our estimates decreases with increasing $[Ca^{2+}]_i$ above ~22 μM $[Ca^{2+}]_i$. The approximate agreement with Levy and Fein (1985) is not entirely expected, since Ca^{2+}-selective electrodes sample $[Ca^{2+}]_i$ from a localized region, whereas aequorin sampled $[Ca^{2+}]_i$ from a wide region.

The resting $[Ca^{2+}]_i$ values we estimate for *Limulus* ventral photoreceptors compare with values in salamander rods of <0.6 μM measured by McNaughton et al. (1986).

Mechanisms Underlying $[Ca^{2+}]_i$ Change

If we consider Ca^{2+} fluxes from Figs. 3 and 4, it seems reasonable to suspect that the transient nature of the rise in $[Ca^{2+}]_i$ induced by Na_o^+ removal (Fig. 3) is a reflection of the particular balance between Ca^{2+} entry and Ca^{2+} removal. Ca^{2+} entry may be due principally to reverse exchange if Ca^{2+} leakage is small. Ca^{2+} release from intracellular stores does not appear to occur in the dark (P. M. O'Day, unpublished results). Ca^{2+} entry from the voltage-dependent conductance (Lisman et al., 1982) is likely to be very small under the conditions of Figs. 3 and 4. The results of Lisman et al. (1982) suggest that at the membrane potential in Fig. 3, a transient voltage-dependent Ca^{2+} current of peak value of 3 nA and ~1 s in duration would result. Assuming that 99.65% of entering Ca^{2+} becomes bound, a rise in $[Ca^{2+}]_i$ of only 0.22 μM would be expected, a small amount in comparison with the observed $[Ca^{2+}]_i$ rise in Fig. 3. We have not ruled out the possibility that the rise in $[Ca^{2+}]_i$ itself triggers Ca^{2+} release, as appears to be the case in cardiac muscle (Endo et al., 1970; Ford and Podolsky, 1970).

Forward Na^+/Ca^{2+} exchange. A Na_o^+-dependent $[Ca^{2+}]_i$ decline was observed in both normal Ca^{2+} (Fig. 3) and 0-Ca^{2+} (Fig. 4) saline. This suggests that the $[Ca^{2+}]_i$ decline was not solely a result of a reduction in Ca^{2+} entry. Thus, there must have been some Ca^{2+} extrusion. The Na^+ dependence of the extrusion is consistent with a role for Na^+/Ca^{2+} exchange.

If we assume that the increase in the rate of $[Ca^{2+}]_i$ decline observed after Na_o^+ restoration in Fig. 3 is due principally to forward exchange, we can estimate roughly the rate of this process. The maximal rate of reduction in $[Ca^{2+}]_i$ that we found was ~1 μM/s (Fig. 5). The maximal estimated forward Na^+/Ca^{2+} exchange rate based on these data would be 70 fmol/s. Assuming a surface area of 4.2×10^{-3} cm^2 for the highly invaginated membrane surface area of the photoreceptor (Lisman and Bering, 1977), we estimate a rate of 16 pmol/(cm^2s) for forward exchange when $[Ca^{2+}]_i = 5.5$ μM. This is quite high in comparison with the rates of Na^+/Ca^{2+} exchange reported for squid axon: 2–3 fmol/(cm^2s) in the 1–10 μM range (DiPolo and Beauge, 1983).

Our observation that Na_o^+-dependent $[Ca^{2+}]_i$ reduction can occur over a wide range of detectable $[Ca^{2+}]_i$ (6 to <0.4 μM) suggests that Na^+/Ca^{2+} exchange may play a physiological role in extruding Ca^{2+} over this $[Ca^{2+}]_i$ range. Na^+/Ca^{2+} exchange in other neurons has been described as a high capacity, low affinity system (DiPolo and Beauge, 1983) operating maximally in the 1–10-μM range of $[Ca^{2+}]_i$. To estimate the Ca^{2+} dependence of the Na^+/Ca^{2+} exchange in *Limulus* ventral photoreceptors, we have replotted the data of Fig. 3, plotting the rate of $[Ca^{2+}]_i$ decline vs. $[Ca^{2+}]_i$. Fig. 10, a plot of the rate of Na_o^+-dependent $[Ca^{2+}]_i$ decline, suggests that forward exchange can operate down to levels near noise levels, but the rate of Ca^{2+} reduction appears optimal above 4 μM. In a normally functioning photoreceptor, maximum forward exchange rates might be expected during illumination when $[Ca^{2+}]_i$ is elevated and $[Na^+]_i$ is kept low by the Na^+/K^+ exchange pump (Brown and Lisman, 1972). The fact that the decline in $[Ca^{2+}]_i$ in 0-Ca^{2+} saline was much slower in the absence of Na_o^+ than in its presence (Fig. 5, phase E vs. E') suggests that, at high $[Ca^{2+}]_i$, Na_o^+-independent Ca^{2+} removal (perhaps a Ca^{2+}-ATPase) is less significant than Na^+/Ca^{2+} exchange.

In contrast, three pieces of evidence suggest that Na^+/Ca^{2+} exchange is not a major factor in Ca^{2+} extrusion at very low $[Ca^{2+}]_i$: (*A*) Fig. 10 suggests that the rate of fractional $[Ca^{2+}]_i$ decline due to Na_o^+ was much smaller at low levels of $[Ca^{2+}]_i$ (<0.5 μM) than at higher levels. (*B*) An electrogenic Na^+/Ca^{2+} exchange process should have a reversal potential at which forward and reverse exchange fluxes are equal and opposite, precluding a net transport of Ca^{2+} (Mullins, 1979). This is described by the equation:

$$E_x = 3E_{Na} - 2E_{Ca} \quad (1)$$

where E_{Na} and E_{Ca} represent the Nernst equilibrium potentials for Na^+ and Ca^{2+}, respectively, and an exchange stoichiometry of 3 Na^+ to 1 Ca^{2+} is assumed (similar to that estimated from vertebrate photoreceptors by Yau and Nakatani, 1984 or Hodgkin et al., 1987). If we assume that the $[Na^+]_i$ is similar to $[Na^+]_i$ in several other neurons, E_{Na} may be ~+55 mV. If we consider the case where a photoreceptor in the dark has a resting potential at which voltage-dependent Ca^{2+} channels

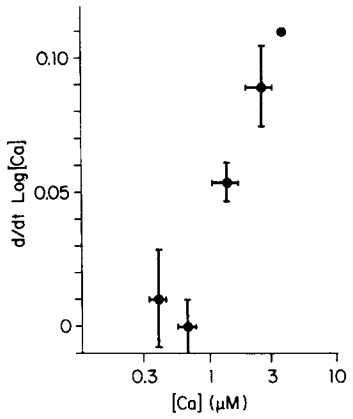

FIGURE 10. $[Ca^{2+}]_i$ dependence of $[Ca^{2+}]_i$ reduction. Rates of $[Ca^{2+}]_i$ reduction due to Na^+ restoration were greater at greater $[Ca^{2+}]_i$. Data from phase *E* of Fig. 3 *C* are plotted vs. the absolute level of estimated $[Ca^{2+}]_i$ (from Fig. 3 *B*). Data were grouped into four ranges of $[Ca^{2+}]_i$. Ordinate is d/dt ($\log_{10}$ molar $[Ca^{2+}]_i$). Error bars depict standard errors of the mean.

are closed (Lisman et al., 1982), e.g., −80 mV, the equation above would yield $[Ca^{2+}]_i$ = 0.5 μM. This suggests that under physiological conditions where there is no Ca^{2+} entry, no Ca^{2+} mobilization, and no active $[Ca^{2+}]_i$ removal, Na^+/Ca^{2+} exchange might equilibrate $[Ca^{2+}]_i$ to roughly 0.5 μM, but not lower. (*C*) But $[Ca^{2+}]_i$ might be reduced below this level by some other process. If the correlation between $[Ca^{2+}]_i$ and light sensitivity (Brown and Lisman, 1972) holds true even at very low $[Ca^{2+}]_i$, then it is likely that there exists a process that can reduce $[Ca^{2+}]_i$ further than we have measured, since light sensitivity can continue to rise in the dark for at least 20 min after a large desensitization (Lisman and Strong, 1979). Yet reductions of $[Ca^{2+}]_i$ in our experiments persist for only a few minutes after desensitization. Alternatively, this gradual rise in sensitivity may be unrelated to $[Ca^{2+}]_i$.

Reverse Na^+/Ca^{2+} exchange. Reverse exchange may be responsible for the transient $[Ca^{2+}]_i$ rises induced by Na_o^+ removal (Figs. 3–6), although we have not ruled out the idea that Ca^{2+} entry might also (or instead) occur by passive leakage in the absence of Na^+-dependent extrusion. Ca^{2+} entry by reverse exchange itself may be

transient because of the consequent increase in $[Ca^{2+}]_i$ and possible reduction of $[Na^+]_i$. Such changes would reduce the driving force for reverse exchange. An example based on Fig. 3 and a model of Na^+/Ca^{2+} exchange with a stoichiometry of 3 Na^+ to 1 Ca^{2+} suggests that Ca^{2+} entry and Na^+ efflux could reduce the driving force for reverse exchange substantially in a short period of time: If $[Ca^{2+}]_i$ rises by 20 μM by reverse exchange, and if this represents 0.35% of entering Ca^{2+}, then $[Na^+]$ must have been reduced by 17 mM by exchange. A rise in $[Ca^{2+}]_i$ of 20 μM may represent a 40-fold or greater change in $[Ca^{2+}]_i$ from resting levels, and this could reduce the driving force for reverse exchange by nearly 100 mV. A similar calculation for $[Na^+]_i$ changes cannot be made in the absence of a $[Na^+]_i$ measurement; however, data of Lisman and Brown (1972) suggest that a 17-mM change in $[Na^+]_i$ can be sufficient to reduce the light sensitivity of *Limulus* ventral photoreceptors significantly. Such a desensitization is probably due to the effects of changing $[Na^+]_i$ on Na^+/Ca^{2+} exchange.

Our observation that Na^+ injection caused a rise in $[Ca^{2+}]_i$ only in the presence of high $[Ca^{2+}]_0$ (Fig. 7) provides strong evidence that reverse exchange can occur. This result supports the argument that Ca^{2+} entry induced by Na^+ removal involves the reversal of Na^+/Ca^{2+} exchange.

We very gratefully acknowledge Drs. Ed Johnson, John Lisman, and Alan Fein for help and advice during the course of the experiments. We thank Drs. Juan Bacigalupo, Deric Bownds, Wes Corson, Judith Eisen, Ed Johnson, Simon Levy, John Lisman, Grant Nicol, Richard Payne, Phyllis Robinson, and Hennig Stieve for helpful discussions and critical comments on the manuscript.

This work was supported by National Science Foundation grant BNS-84024463 and a grant from the Medical Research Foundation of Oregon.

Original version received 27 January 1988 and accepted version received 26 September 1988.

REFERENCES

Allen, D. G., and J. R. Blinks. 1978. Calcium transients in aequorin-injected frog cardiac muscle. *Nature.* 273:509–513.

Bader, C. R., F. Baumann, and J. Bertrand. 1976. The role of intracellular calcium and sodium in light-adaptation in the retina of the honeybee drone (*Apis mellofera*). *Journal of General Physiology.* 67:475–491.

Baker, P. F., M. P. Blaustein, A. L. Hodgkin, and R. A. Steinhardt. 1969. The influence of calcium on sodium efflux in squid axons. *Journal of Physiology.* 200:431–458.

Baker, P. F., and R. DiPolo. 1984. Axonal calcium and magnesium homeostasis. *Current Topics in Membranes and Transport.* 22:195–247.

Bastian, B. L., and G. L. Fain. 1982. The effects of low calcium and background light on the sensitivity of toad rods. *Journal of Physiology.* 330:307–329.

Blaustein, M. P. 1977. Effects of internal and external cations and of ATP on sodium-calcium and calcium-calcium exchange in squid axons. *Biophysical Journal.* 20:79–111.

Blaustein, M. P. 1984. The energetics and kinetics of sodium-calcium exchange in barnacle muscles, squid axons, and mammalian heart: the role of ATP. *In* Electrogenic Transport: Fundamental Principles and Physiological Implications. M. P. Blaustein and M. Lieberman, editors. Raven Press, New York. 129–147.

Blaustein, M. P., and A. L. Hodgkin. 1969. The effect of cyanide on the efflux of calcium from squid axons. *Journal of Physiology.* 400:497–527.

Blinks, J. R., W. G. Wier, P. Hess, and F. G. Prendergast. 1982. Measurement of Ca^{2+} concentrations in living cells. *Progress in Biophysics and Molecular Biology.* 40:1–114.

Bolsover, S. R., and J. E. Brown. 1985. Calcium ion, an intracellular messenger of light-adaptation, also participates in excitation of *Limulus* photoreceptors. *Journal of Physiology.* 364:381–393.

Brown, H. M., and B. Rydqvist. 1982. Intracellular free Mg^{2+} in *Balanus* photoreceptors estimated with eriochrome blue. *Biophysical Journal.* 37:196*a*. (Abstr.)

Brown, J. E., and J. R. Blinks. 1974. Changes in intracellular free calcium concentration during illumination of invertebrate photoreceptors. *Journal of General Physiology.* 64:643–665.

Brown, J. E., P. K. Brown, and L. H. Pinto. 1977. Detection of light-induced changes of intracellular ionized calcium concentration in *Limulus* ventral photoreceptors using Arsenazo III. *Journal of Physiology.* 267:299–320.

Brown, J. E., and J. E. Lisman. 1972. An electrogenic sodium pump in *Limulus* ventral photoreceptor cells. *Journal of General Physiology.* 59:720–740.

Brown, J. E. and J. E. Lisman. 1975. Intracellular calcium modulates sensitivity and time scale in *Limulus* ventral photoreceptors. *Nature.* 258:252–254.

Brown, J. E., and M. I. Mote. 1974. Ionic dependence of reversal voltage of the light response in *Limulus* ventral photoreceptors. *Journal of General Physiology.* 63:337–350.

Celio, M. R., and C. W. Heizmann. 1981. Calcium-binding protein parvalbumin as a neuronal marker. *Nature.* 293:300–302.

Corson, D. W., and A. Fein. 1983. Quantitative pressure injection of picoliter volumes into *Limulus* ventral photoreceptors. *Biophysical Journal.* 44:299–304.

DiPolo, R. 1973. Calcium efflux from internally dialyzed squid giant axons. *Journal of General Physiology.* 62:575–589.

DiPolo, R. 1979. Calcium influx in internally dialyzed squid giant axons. *Journal of General Physiology.* 73:91–113.

DiPolo, R., and L. Beauge. 1983. The calcium pump and sodium-calcium exchange in squid axons. *Annual Reviews of Physiology.* 45:313–324.

DiPolo, R., and L. Beauge. 1987. Characterization of the reverse Na/Ca exchange in squid axons and its modulation by Ca_i and ATP. *Journal of General Physiology.* 90:505–525.

Dodge, F. A., B. W. Knight, and J. Toyoda. 1968. Voltage noise in *Limulus* visual cells. *Science.* 160:88–90.

Ebashi, S. 1980. Regulation of muscle contraction. *Proceedings of the Royal Society of London Series B.* 207:259–286.

Endo, M., M. Tanaka, and Y. Ogawa. 1970. Calcium induced release of calcium from sarcoplasmic reticulum of skinned skeletal muscle fibres. *Nature.* 228:34–36.

Fein, A., and J. S. Charlton. 1977. A quantitative comparison of the effects of intracellular calcium injection and light adaptation on the light response of *Limulus* ventral photoreceptors. *Journal of General Physiology.* 70:591–600.

Ford, L. E., and R. J. Podolsky. 1970. Regenerative calcium release within muscle cells. *Science.* 167:58–59.

Harary, H. H., and J. E. Brown. 1984. Spatially non-uniform changes in intracellular calcium ion concentration. *Science.* 225:292–294.

Hodgkin, A. L. 1951. The ionic basis of electrical activity in nerve and muscle. *Biological Review.* 26:339–409.

Hodgkin, A. L., P. A. McNaughton, and B. J. Nunn. 1987. Measurement of sodium-calcium exchange in salamander rods. *Journal of Physiology.* 391:347–370.

Keller, M. P., and P. M. O'Day. 1987. Separation of "forward" and "reverse" Na^+/Ca^{2+}-exchange in *Limulus* ventral photoreceptors. *Investigative Ophthalmology and Visual Science.* 27(Suppl.):284. (Abstr.)

Levy, S., and A. Fein. 1985. Relationship between light sensitivity and intracellular free Ca concentration in *Limulus* ventral photoreceptors. *Journal of General Physiology.* 85:805–841.

Lisman, J. E., and H. Bering. 1977. Electrophysiological measurement of the number of rhodopsin molecules in single *Limulus* photoreceptors. *Journal of General Physiology.* 70:621–633.

Lisman, J. E., and J. E. Brown. 1972. The effects of intracellular iontophoretic injection of calcium and sodium ions on the light response of *Limulus* ventral photoreceptors. *Journal of General Physiology.* 59:701–719.

Lisman, J. E., and J. E. Brown. 1975. Light-induced changes of sensitivity in *Limulus* ventral photoreceptors. *Journal of General Physiology.* 66:473–488.

Lisman, J. E., G. L. Fain, and P. M. O'Day. 1982. Voltage-dependent conductances in *Limulus* ventral photoreceptors. *Journal of General Physiology.* 79:187–209.

Lisman, J. E., and J. A. Strong. 1979. The initiation of excitation and light adaptation in *Limulus* ventral photoreceptors. *Journal of General Physiology.* 73:219–243.

McNaughton, P. A., L. Cervetto, and B. J. Nunn. 1986. Measurement of the intracellular free calcium concentration in salamander rods. *Nature.* 322:261–263.

Millecchia, R., and A. Mauro. 1969*a*. The ventral photoreceptor cells of *Limulus*. II. The basic photoresponse. *Journal of General Physiology.* 54:310–330.

Millecchia, R., and A. Mauro. 1969*b*. The ventral photoreceptor cells of *Limulus*. III. A voltage-clamp study. *Journal of General Physiology.* 54:331–351.

Minke, B., and M. Tsacopoulos. 1986. Light-induced sodium-dependent accumulation of calcium and potassium in the extracellular space of bee retina. *Vision Research.* 26:679–690.

Moisescu, D. G., and C. C. Ashley. 1977. The effect of physiologically occurring cations upon aequorin light emission: determination of binding constants. *Biochimica et Biophysica Acta.* 460:189–205.

Mullins, L. J. 1979. The generation of electric currents in cardiac fibers by Na^+/Ca^{2+}-exchange. *American Journal of Physiology.* 236:C103–C110.

Mullins, L. J., and F. J. Brinley, Jr. 1975. Sensitivity of calcium efflux from squid axons to changes in membrane potential. *Journal of General Physiology.* 65:135–152.

Mullins, L. J., and J. Requena. 1981. The "late" Ca channels in squid axons. *Journal of General Physiology.* 78:683–700.

Nagy, K., and H. Stieve. 1983. Changes in intracellular free calcium ion concentration, in the course of dark-adaptation measured by arsenazo III in the *Limulus* photoreceptors. *Biophysics of Structure and Mechanism.* 9:207–223.

O'Day, P. M., and M. P. Keller. 1987. Evidence for electrogenic Na^+/Ca^{2+}-exchange in *Limulus* ventral photoreceptors. *Biophysical Journal.* 51:273*a*. (Abstr)

O'Day, P. M., J. E. Lisman, and M. Goldring. 1982. Functional significance of voltage-dependent conductances in *Limulus* ventral photoreceptors. *Journal of General Physiology.* 79:211–232.

Payne, R., D. W. Corson, and A. Fein. 1986. Pressure injection of calcium both excites and adapts *Limulus* ventral photoreceptors. *Journal of General Physiology.* 88:107–126.

Payne, R., and A. Fein. 1987. Inositol 1,4,5 trisphosphate releases calcium from specialized sites within *Limulus* photoreceptors. *Journal of Cell Biology.* 104:933–937.

Reuter, H., and N. Seitz. 1968. The dependence of calcium efflux from cardiac muscle on temperature and external ion composition. *Journal of Physiology.* 195:451–470.

Schnetkamp, P. P. M. 1986. Sodium-calcium exchange in the outer segments of bovine rod photoreceptors. *Journal of Physiology.* 373:25–45.

Waloga, G., J. E. Brown, and L. H. Pinto. 1975. Detection of changes in $[Ca^{2+}]_i$ from *Limulus* ventral photoreceptors using Arsenazo III. *Biological Bulletin.* 149:449–450.

Walz, B., A. Fein. 1983. Evidence for calcium-sequestering smooth ER in *Limulus* ventral photoreceptors. *Investigative Ophthalmology and Visual Science.* 24(Suppl.):281. (Abstr.)

Wood, J. G., R. W. Wallace, and W. Y. Cheung. 1980. Immunocytochemical studies of the localization of calmodulin and CaM-BP_8O in brain. *Calcium and Cell Function.* 1:291–303.

Yau, K. W., and K. Nakatani. 1984. Electrogenic Na^+/Ca^{2+}-exchange in retinal rod outer segment. *Nature.* 331:661–663.