Transduction Persists in Rod Photoreceptors after Depletion of Intracellular Calcium

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ABSTRACT We have examined the role of Ca^{++} in phototransduction by manipulating the intracellular Ca^{++} concentration in physiologically active suspensions of isolated and purified rod photoreceptors (OS-IS). The results are summarized by the following. (*a*) Measurement of Ca^{++} content using arsenazo III spectroscopy demonstrates that incubation of OS-IS in 10 nM Ca^{++}-Ringer's solution containing the Ca^{++} ionophore A23187 reduces their Ca^{++} content by 93%, from 1.3 to 0.1 mol Ca^{++}/mol rhodopsin. Virtually the same reduction can be accomplished in 10 nM Ca^{++}-Ringer's without ionophore, presumably via the plasma membrane Na/Ca exchange mechanism. (*b*) Hundreds of photoresponses can be obtained from the Ca^{++}-depleted OS-IS for at least 1 h in 10 nM Ca^{++}-Ringer's with ionophore. The kinetics and light sensitivity of the photoresponse are essentially the same in the presence or absence of the ionophore in 10 nM Ca^{++}. (*c*) The addition of A23187 in 1 mM Ca^{++}-Ringer's results in a Ca^{++} influx that rapidly suppresses the dark current and the photoresponse. This indicates that there is an intracellular site at which Ca^{++} can modulate the light-regulated conductance. Both the current and photoresponse can be restored if intracellular Ca^{++} is reduced by lowering the external Ca^{++} to 10 nM. During the transition from high to low Ca^{++}, the response duration becomes shorter, which suggests that it can be regulated by a Ca^{++}-dependent mechanism. (*d*) If the dark current and the photoresponse are suppressed by adding A23187 in 1 mM Ca^{++}-Ringer's, the subsequent addition of the cyclic GMP phosphodiesterase inhibitor isobutylmethylxanthine can restore the current and photoresponse. This implies that under conditions where the rod can no longer control its intracellular Ca^{++}, the elevation of cyclic GMP levels can restore light regulation of the channels. The persistence of normal flash responses under conditions where intracellular Ca^{++} levels are reduced and perturbed suggests that changes in the intracellular Ca^{++} concentration do not cause the closure of the light-regulated channel.

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INTRODUCTION

Visual transduction in vertebrate rod photoreceptors is believed to be regulated by a diffusible internal messenger that mediates between the excitation of rhodopsin in the disk membranes and the closure of ionic channels in the plasma membrane (Baylor and Fuortes, 1970). The most influential models for visual transduction have suggested that the internal messenger is either Ca (Yoshikami and Hagins, 1971; Hagins, 1972) or cyclic GMP (Bitensky et al., 1971; Woodruff et al., 1977; Miller 1981). For reviews of the excitation process and the nature of the internal messenger, refer to Kaupp and Schnetkamp (1982) and Korenbrot (1985).

In the cyclic GMP hypothesis, light isomerizes rhodopsin, and this bleached rhodopsin (Rh*) activates a sequence of enzymatic reactions that ultimately reduces the intracellular concentration of cyclic GMP and results in channel closure. The decrease of cyclic GMP upon illumination precedes suppression of the dark current, which suggests that it is sufficiently rapid to regulate the membrane permeability (Cote et al., 1984). Intracellular injection of cyclic GMP produces membrane depolarization (Nicol and Miller, 1978) and a large increase in the membrane current (MacLeish et al., 1984). Recent studies have demonstrated large increases in the dark current and suppression of the current by light when cyclic GMP is perfused intracellularly via a patch pipette (Cobbs and Pugh, 1985; Matthews et al., 1985; Zimmerman et al., 1985) or applied to a truncated rod outer segment (Yau and Nakatani, 1985*b*). Cyclic GMP has also been shown to open a conductance in an excised patch of rod membrane (Fesenko et al., 1985; Zimmerman et al., 1985) as well as in the disk membrane (Carretta and Cavaggioni, 1983; Caretta, 1985; Koch and Kaupp, 1985).

In the Ca hypothesis, Ca^{++} released by light from storage sites inside the disks diffuses to the plasma membrane and closes ionic channels through direct binding. In accordance with this idea, raising or lowering the extracellular Ca^{++} concentration (presumed to concomitantly raise/lower intracellular Ca^{++}) hyperpolarizes or depolarizes, respectively, the rod membrane (Brown and Pinto, 1974; Bastian and Fain, 1979, 1982) and also affects the current flowing in the dark (Yoshikami and Hagins, 1973; Yau et al., 1981; Hodgkin et al., 1984). The intracellular injection of Ca^{++} or EGTA results in membrane hyperpolarization or depolarization, respectively (Brown et al., 1977), and changes the magnitude of the dark current (MacLeish et al., 1984), as if the reduction of the internal messenger level had enhanced the light-regulated conductance. The state of light adaptation is also influenced by the Ca^{++} level. Lowering the external Ca^{++} concentration causes desensitization of the light response so that a greater stimulus intensity is required to close the additional channels opened by the reduced concentration of internal messenger (Yoshikami and Hagins, 1973; Bastian and Fain, 1979).

However, recent results have shown that Ca^{++} can flow through the light-regulated conductance without suppressing it (Yau and Nakatani, 1984; Hodgkin et al., 1984, 1985). Also, intracellular perfusion of the Ca buffer BAPTA does not lead to suppression of the light response, as might be expected from the Ca hypothesis (Matthews et al., 1985). Biochemical measurements have not dem-

onstrated light-induced Ca^{++} release from isolated disks (Smith et al., 1977; Smith and Bauer, 1979) or lysed or intact rods (Szuts and Cone, 1977; Kaupp et al., 1979*a*; Schnetkamp and Kaupp, 1983) with stoichiometries greater than ~1 Ca^{++} released per bleached rhodopsin, which is far below the value calculated to achieve the required amplification (Cone, 1973).

In this study, we have used purified suspensions of physiologically active rod outer segments that remain attached to their mitochondria-rich inner segments (Biernbaum and Bownds, 1985). This permits spectroscopic monitoring of Ca^{++} movements caused by the addition of Ca ionophores or buffers, measurements that could not be made in previous studies using intact retinas (Hagins and Yoshikami, 1974; Bastian and Fain, 1979). Electrophysiological properties of individual rods could be correlated with spectroscopic Ca^{++} measurements on cellular suspensions under identical conditions. Ca ionophores and buffers were used to manipulate internal Ca^{++} levels (Kaupp et al., 1979*b*; Schnetkamp, 1979; Lew and Garcia-Sancho, 1985). These experiments show that changes in Ca^{++} levels are not likely to be responsible for the initial closure of light-regulated channels upon illumination, and that inhibition by Ca^{++} of the channel closure mechanism can be overcome by raising internal cyclic GMP levels. A preliminary account of parts of this work has appeared elsewhere (Nicol et al., 1985).

MATERIALS AND METHODS

Preparation of Frog OS-IS

Suspensions of rod outer segments that retain the mitochondria-rich ellipsoid portion of their inner segments (OS-IS) were obtained from dark-adapted retinas of the bullfrog, *Rana catesbeiana* (Biernbaum and Bownds, 1985). Briefly, isolated retinas were gently shaken in what we shall define as the normal Ringer's solution containing (mM): 5% Percoll (vol/vol), 105 NaCl, 2 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 5 $NaHCO_3$, 5 glucose, and 10 HEPES, buffered at pH 7.5. Solutions were oxygenated before use. After being shaken, the retinas were shredded into fine particles with forceps. After the particles settled, the suspension was layered onto a continuous Percoll gradient (30–70% Percoll in Ringer's). Gradients were centrifuged at 4,000 rpm for 5 min. Three bands could be distinguished. The upper band contained cellular debris with a low rhodopsin content. The middle band consisted predominantly of outer segments (OS) with an osmotically leaky plasma membrane. The lower band contained intact OS and OS-IS and was typically between 65 and 90% OS-IS. All experiments were done at room temperature. The rhodopsin concentration was determined by difference spectroscopy (Bownds et al., 1971).

Electrophysiology

An aliquot (3–5 μl) from a suspension of frog OS-IS in 5% Percoll-Ringer's containing 1 mM Ca^{++} was placed in a recording chamber (approximate volume, 350 μl). The inner segment of an OS-IS was drawn into a suction electrode under visual control in the dark using an infrared video system. The current passing from the inner to the outer segment was measured with a current-to-voltage converter (Baylor et al., 1979). The OS-IS was illuminated with a small focused spot (75 μm diam) whose intensity was controlled with a series of neutral density filters (calibrated with a Cary 14 spectrophotometer, Varian Instruments, Palo Alto, CA). The duration of the flash was 12 ms, as measured with a photodiode. All current records were low-pass-filtered at 20 Hz with an eight-pole active

filter (Khron-Hite Corp., Avon, MA). Current traces were either recorded on a chart recorder (Gould, Inc., Cleveland, OH) or digitized (sampling interval, 10 ms) and stored on computer disk memory. The recording chamber could be perfused with different Ringer's solutions. The inflow was controlled with a six-way valve whose change in position was recorded on the chart recorder. The effluent was removed by a vacuum pump. The time course of solution changes (10–15 s) was judged from the junctional current resulting from the ethanol in which the ionophore was dissolved, as shown in Fig. 8*A*.

The extent of rhodopsin bleaching was determined in the following manner. An aliquot from a suspension of rod outer segments was placed into the recording chamber. Separate suspensions were illuminated for different times with a large diffuse spot covering the entire chamber. The suspension was removed and the amount of bleached rhodopsin as compared with a dark sample was determined by absorbance measurements using a Cary 14 spectrophotometer. An OS-IS suspension was then placed in the chamber, the inner segment was drawn into a suction pipette, and the flash intensity-response amplitude relation was determined using the same large diffuse illumination. Thus, the OS-IS response curve could be calibrated against the spectroscopic measurement of rhodopsin bleaching. The spot was focused down to 75 μm as it passed through the microscope condenser. The flash intensity response was measured again with the small spot. The intensity-response curve for the large diffuse illumination was used to calibrate the intensity-response curve for the small focused spot. It was determined that for the small focused spot, ~30 bleached rhodopsins (Rh*) gave a half-maximal photoresponse. The uncertainty in the absorbance measurements of rhodopsin bleaching was determined to be 5%.

Measurements of Ca^{++} Release from Intact Rods

The release of Ca^{++} from suspensions of either OS-IS or OS by A23187 was followed spectroscopically with the Ca^{++}-indicating dye arsenazo III (Kaupp et al., 1979*b*; Koch and Kaupp, 1985). Three separate protocols were used to purify OS-IS in different media. First, frog OS-IS were purified on a normal Percoll-Ringer's gradient. OS-IS were then gently pelleted and resuspended in Ringer's solution (no added Ca^{++} or Mg^{++}) where Na^+ had been replaced by K^+ to prevent depletion of intracellular Ca^{++} by Na/Ca exchange (Schnetkamp, 1980). Second, OS-IS were purified on a Percoll-Ringer's gradient that contained no added Ca^{++} or Mg^{++} (<10 μM). OS-IS were used without further resuspension. Finally, OS-IS were purified on a Percoll-Ringer's gradient that contained no added Ca^{++} or Mg^{++} and Na^+ was replaced by tetramethylammonium (TMA^+).

Aliquots of these OS-IS suspensions were mixed with their respective Ringer's solutions containing arsenazo III. The concentrations in the cuvette were: 50 μM arsenazo III, 3–6 μM Ca^{++}, 1–2 μM rhodopsin, 115 mM of either NaCl, KCl, or TMA-Cl, 5 mM glucose, and 10 mM HEPES at pH 7.5. The suspension in the cuvette was gently agitated with a small stir bar. The release of Ca^{++} from OS-IS resulting from the addition of A23187 was measured in an Aminco DW2a spectrophotometer (SLM Instruments, Inc., Urbana, IL), recording the difference in absorbance at 650 and 730 nm with a spectral bandwidth of 10 nm. A23187 was added to the cuvette by rapid injection of a 1 mM stock solution. Mixing was complete within 3 s. Ca-indicating absorbance changes were calibrated by the addition of known amounts of Ca^{++} to the same cuvette.

Measurements of Total Ca Content

OS-IS purified on a normal Percoll-Ringer's gradient were incubated in the dark for various times in an Na^+-Ringer's of different Ca^{++} and A23187 concentrations. All

experimental manipulations were performed in the dark using an infrared image converter. OS-IS suspensions were centrifuged at 7,000–15,000 rpm for 10 s. The pellet, without resuspension, was gently rinsed twice with 1 ml of TMA^+-Ringer's solution containing no added Ca^{++} or Mg^{++}. Between rinses, the incubation tube was carefully wiped with cotton swabs that had been washed in double-distilled water and dried to remove any residual Ca^{++}. Pellets were then dissolved overnight in 1 ml of a medium containing 50 μM arsenazo III, 1.5% Triton X-100, and 10 mM HEPES at pH 7.0. Before use, the arsenazo III–solubilizing solution was passed over a Chelex-100 column to remove residual Ca^{++}. Total Ca^{++} in the dissolved pellet was determined from difference spectra recorded between 400 and 750 nm with a spectral bandwidth of 3 nm. Ca^{++} content was calculated from the absorbance at 650 nm by comparison with standard spectra of the same medium with known Ca^{++} concentrations. Control experiments demonstrated that the solution remaining in the aqueous volume of the pellet was <0.01% of the total suspension volume.

Partitioning of A23187

The distribution of A23187 into frog OS-IS membranes was determined by incubation of an OS-IS suspension (1.5 μM rhodopsin) with A23187 (20 μM) for 5 min. The suspension was centrifuged and the concentration of A23187 remaining in the supernatant was determined from the ultraviolet spectrum (200–400 nm). Approximately 50% of the A23187 partitioned into the membranes. This corresponds to a stoichometry of 6 mol A23187/mol rhodopsin; for an intact OS-IS, this would be equivalent to ~18 mM A23187.

Previous studies, unlike the results described below, were unable to show an effect of Ca ionophores at 1 mM Ca^{++}, which indicated that A23187 and X537A complexed with Ca^{++} and were only effective when used at lowered Ca^{++} concentrations (Hagins and Yoshikami, 1974; Bastian and Fain, 1979). We have examined this point and found that the extent of A23187 incorporation into intact frog OS-IS was similar regardless of the Ca^{++} concentration. Using the method described above, at 1 mM Ca^{++}, 6.4 ± 0.1 mol A23187/mol rhodopsin was incorporated into the rod membranes and at 10 nM Ca^{++}, 6.7 ± 0.2 mol A23187/mol rhodopsin (n = 5) was incorporated.

Solutions

A23187 and ionomycin were dissolved in ethanol as either 1 or 5 mM stock solutions added to the Ringer's solution just before use. The Ca^{++} content of the A23187 stock solution was determined to be 0.05 μM Ca^{++}/μM A23187 by arsenazo III differential spectroscopy. Isobutylmethylxanthine (IBMX) was used as a 2 mM stock solution dissolved in normal Ringer's. The 10 nM Ca^{++} used for electrophysiological measurements was obtained by adding 0.39 mM EGTA to 0.1 mM Ca^{++}, 5% Percoll-Ringer's, pH 7.5 (Caldwell, 1970). All solutions for arsenazo III spectroscopy were made with double-distilled water.

RESULTS

These experiments make use of purified suspensions of frog rod outer segments that remain attached to the mitochondria-rich ellipsoid portion of their inner segments (OS-IS, Biernbaum and Bownds, 1985). OS-IS maintain normal electrophysiological activity for several hours and can be prepared in sufficient quantity to allow the measurement of Ca^{++} fluxes and Ca^{++} content. This makes possible the direct measurement of the Ca^{++} depletion that occurs when these cells are incubated in low-Ca^{++} media containing Ca^{++} ionophores, and permits

correlation with electrophysiological measurements made under similar conditions.

Kinetics of A23187-induced Ca^{++} Release

This section describes the use of arsenazo III spectroscopy to measure the Ca^{++} content of OS-IS under different conditions. The addition of A23187 to a suspension of frog OS-IS can cause the rapid release of Ca^{++} from intracellular stores, but the Ca^{++} content depends upon the composition of the external medium. Fig. 1*A* demonstrates the release of Ca^{++} from OS-IS purified on a normal Percoll-Ringer's gradient. The cells were gently pelleted and then resus-

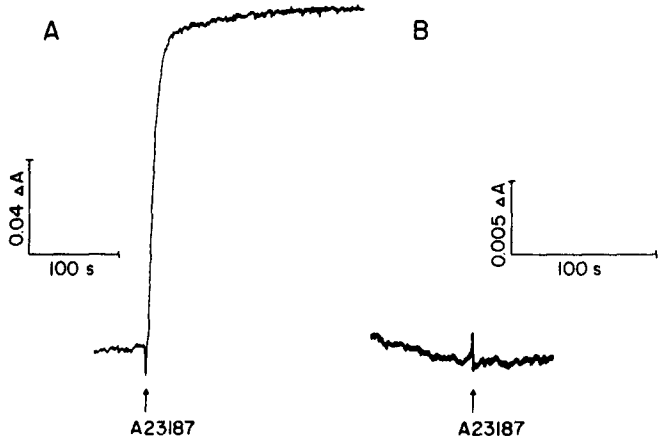

FIGURE 1. Spectroscopic measurement of Ca^{++} release from frog OS-IS using the indicator dye arsenazo III. The difference in absorbance at 650 and 730 nm was recorded, with a spectral bandwidth of 10 nm. In *A*, rods (70% OS-IS) from a 1 mM Ca^{++} Percoll-Ringer's gradient were gently pelleted and resuspended in a K^+-Ringer's, as described in Materials and Methods. The addition of 5 μM A23187 (at the arrow) to the OS-IS suspension caused the rapid release of Ca^{++}. Here the absorbance change corresponded to 1.1 μM Ca^{++} or a release of 1.2 mol Ca^{++}/mol rhodopsin. Similar results were obtained with OS-IS purified on a Percoll-Ringer's gradient where Na^+ was replaced by TMA^+, thus avoiding the resuspension step. In *B*, OS-IS were purified on a normal Percoll-Ringer's gradient with no added Ca^{++} or Mg^{++}, and were not resuspended. The addition of 5 μM A23187 to the OS-IS suspension (75% OS-IS, 1.4 μM rhodopsin) did not cause the release of Ca^{++}.

pended in K^+-Ringer's containing no added Ca^{++} or Mg^{++}. The replacement of Na^+ by K^+ was used to prevent any loss of intracellular Ca^{++} that may have occurred by Na/Ca exchange (Schnetkamp, 1980). The addition of 5 μM A23187 to the cuvette resulted in the rapid release of 1.1 ± 0.1 mol Ca^{++}/mol rhodopsin (n = 4, mean ± SD). The same result was obtained if OS-IS were purified on a Percoll-Ringer's gradient containing no added Ca^{++} or Mg^{++} in which Na^+ was replaced by TMA^+ (data not shown). This avoided resuspension of the OS-IS in an Na^+-free Ringer's. Also, the similarity of these two results implies that the release of Ca^{++} is not dependent on the membrane voltage since the OS-IS membrane will depolarize in the K^+ medium and hyperpolarize in the TMA^+

medium. Fig. 1*B* shows that OS-IS is prepared in Na^+-Ringer's with no added Ca^{++} released little or no Ca^{++} upon ionophore addition, which is consistent with the notion that Na/Ca exchange is capable of reducing intracellular Ca^{++} stores even in the absence of A23187.

The results shown in Fig. 1*B* present the possibility that in the presence of Na^+ and low Ca^{++} concentrations, A23187 may not release the stores of intra-

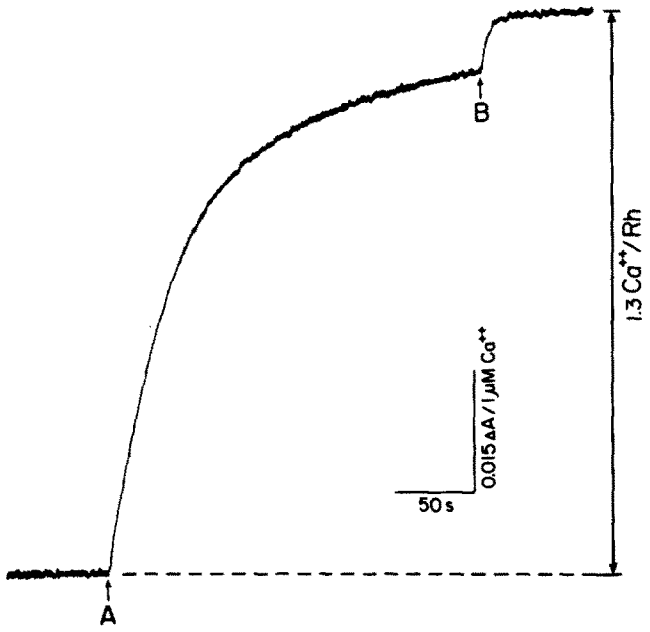

FIGURE 2. Release of Ca^{++} from an OS-IS suspension by A23187 in the presence of extracellular Na^+. OS-IS were isolated and gradient-purified in normal Na^+ Percoll-Ringer's containing 1 mM Ca^{++}. Cells were then gently pelleted and resuspended in 125 mM TMA-Cl with no added Ca^{++} or Mg^{++}. Measurement of the release of Ca^{++} was the same as that described for Fig. 1, except that the cuvette contained 100 μM arsenazo III. At point *A*, 100 μl of 1 M NaCl (spectroscopic grade) was added to the cuvette, making the concentration of Na^+ 50 mM. The addition of Na^+ caused the release of intracellular Ca^{++}, as indicated by the increase in absorbance at 650 nm. At point *B*, 2 μl of 2 mM A23187 (2 μM) was added to the cuvette and caused the release of additional Ca^{++}. The rhodopsin concentration in the cuvette was 3.5 μM, corresponding to a total Ca^{++} release of 1.3 mol Ca^{++}/mol rhodopsin.

cellular Ca^{++}. The efficacy of A23187 in releasing Ca^{++} under these conditions was examined and the results are shown in Fig. 2. OS-IS were isolated and purified in a normal Na^+-Ringer's containing 1 mM Ca^{++} and then resuspended in a TMA^+ medium with no added Ca^{++} or Mg^{++}. At point *A* in the figure, the Na^+-stimulated Ca^{++} release, i.e., Na/Ca exchange, was initiated with the addition of 50 mM NaCl to the cuvette. After the initial rapid phase, the release of Ca^{++} began to slow as the Na/Ca exchange mechanism reached equilibrium (4.1 μM

Ca^{++}; cf. Schnetkamp, 1986). At point *B*, the injection of 2 μM A23187 caused the release of additional Ca^{++} (0.5 μM) from the suspension. The total Ca^{++} released (Na^+ plus A23187) was 1.3 mol Ca^{++}/mol rhodopsin, which agrees with the values shown in Figs. 1*A*, 3, and 4. These results are consistent with the idea that the Na^+-stimulated Ca^{++} efflux can represent a large portion of the total intracellular Ca^{++} and that the Na/Ca exchange mechanism can efficiently deplete the OS-IS of their intracellular stores of Ca^{++}. Also, A23187 can deplete the OS-IS of additional intracellular Ca^{++}, providing the Na/Ca exchanger has not done so.

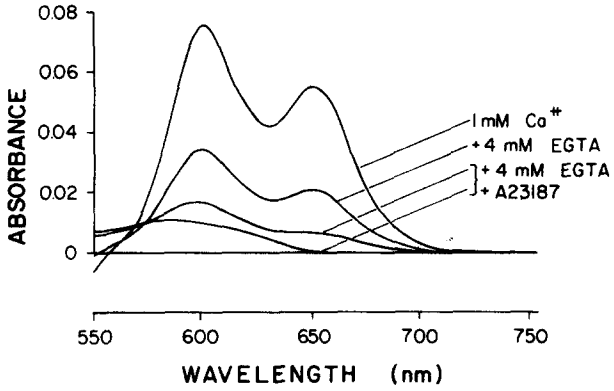

FIGURE 3. Measurement of total Ca^{++} concentration in OS-IS membrane pellets using arsenazo III difference spectroscopy. OS-IS were purified on a normal Percoll-Ringer's gradient. Aliquots (500 μl) of this suspension were added to tubes containing either 1 mM Ca^{++}-Ringer's, 1 mM Ca^{++}-Ringer's plus 4 mM EGTA, or 1 mM Ca^{++}-Ringer's plus 4 mM EGTA plus 20 μM A23187 and incubated for 30 min in the dark. The suspension was then treated and analyzed for Ca^{++} as described in Materials and Methods.

Measurements of Total Ca^{++} Concentration in OS-IS

Fig. 3 shows spectroscopic data of the sort used to evaluate the Ca^{++} content of OS-IS after different treatments. OS-IS were transferred from a common suspension and incubated in three different Ringer's solutions (1 mM Ca^{++}, 1 mM Ca^{++} plus 4 mM EGTA, yielding 10 nM free Ca^{++}, and 1 mM Ca^{++} plus 4 mM EGTA plus A23187) for 30 min in the dark. OS-IS were centrifuged to form pellets; the pellets were washed twice without resuspension and then dissolved in a solution containing arsenazo III, HEPES, and Triton X-100. The absorbance at 650 nm was used to determine the Ca^{++} content of the dissolved pellets. Ca^{++} contents for the experiment shown were 1.6 (top spectrum), 0.5 (middle spectrum), and 0.0 and 0.2 (bottom two spectra) mol Ca^{++}/mol rhodopsin. (The last two values were obtained on two separate OS-IS suspensions.) This illustrates the reduction of endogenous Ca^{++} upon lowering the external Ca^{++} to 10 nM, and the further reduction observed upon addition of ionophore.

The results from a number of experiments are summarized in Fig. 4. OS-IS incubated in 1 mM Ca^{++}-Ringer's had a total Ca^{++} content of 1.3 ± 0.4 mol

Ca^{++}/mol rhodopsin (n = 19, mean ± SD) in experiments where the OS-IS content varied between 60 and 80%. (This value for total Ca^{++}, determined for sedimented OS-IS, is essentially the same as the amount of Ca^{++} released upon addition of A23187 to a suspension of Ca^{++} in K^+-Ringer's [see Fig. 1*A*], which demonstrates that the ionophore is capable of releasing most, if not all, the Ca^{++} and that resuspension of the OS-IS in K^+-Ringer's does not result in a loss of internal Ca^{++}.) The Ca^{++} content of frog OS-IS is consistent with many previous measurements of total rod Ca^{++} that have used a variety of analysis techiques (bovine: Schnetkamp, 1979; Caretta, 1985; frog: Hagins and Yoshikami, 1975; toad: Schroder and Fain, 1984; Fain and Schroder, 1985), but differ from the

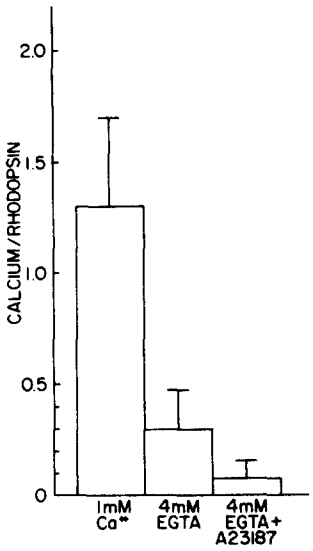

FIGURE 4. Summary of the data on the total Ca^{++} content of OS-IS. Arsenazo III spectra of the type shown in Fig. 3 were used to determine the total Ca^{++} content of OS-IS under different incubation conditions. OS-IS were incubated in the dark for 30 min in either 1 mM Ca^{++}-Ringer's, 1 mM Ca^{++}-Ringer's plus 4 mM EGTA, or 1 mM Ca^{++}-Ringer's plus 4 mM EGTA plus 20 μM A23187. Histograms represent means ± SD.

0.1 mol Ca^{++}/mol rhodopsin reported by Somlyo and Walz (1985). Their value is more characteristic of our Ca^{++}-depleted preparations. The total Ca^{++} measured in outer segment preparations was about the same as that in OS-IS, 1.2 ± 0.1 mol Ca^{++}/mol rhodopsin (n = 4).

When the external Ca^{++} concentration was buffered to 10 nM (1 mM Ca^{++} plus 4 mM EGTA), the total Ca^{++} content was reduced to 0.3 ± 0.2 mol Ca^{++}/mol rhodopsin (n = 4), which is 23% of that measured in 1 mM Ca^{++}. Incubation with 20 μM A23187 and 4 mM EGTA gave total Ca^{++} values of 0.1 ± 0.1 mol Ca^{++}/mol rhodopsin (n = 8), a 93% depletion of the intracellular Ca^{++} measured in 1 mM Ca^{++}. The mean values for the Ca^{++} content in EGTA with or without A23187 are not significantly different at a 95% confidence level; using the

unpaired *t* test. This datum reinforces the implications of Figs. 1*B* and 2 that in a Ringer's of normal Na^+ and low Ca^{++}, the Na/Ca exchange mechanism is probably responsible for the depletion of intracellular Ca^{++}, even in the absence of A23187.

Photoresponses in OS-IS Depleted of Ca^{++}

Previous studies have demonstrated that lowering the extracellular Ca^{++} concentration transiently enhances the light-regulated conductance and desensitizes the photoresponse (see Introduction). This section describes the consequences of lowering the cytoplasmic Ca^{++} content on the dark current and photoresponse recorded from OS-IS.

Fig. 5 illustrates the responses obtained before and after the addition of A23187 to OS-IS maintained in 10 nM Ca^{++}. OS-IS were suspended in 10 nM Ca^{++}-Ringer's for 5 min before drawing the inner segment into the electrode, which ensured that desensitization by low Ca^{++} was largely complete. The

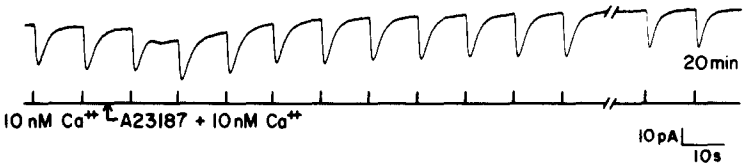

FIGURE 5. Persistence of the photoresponse after the addition of A23187 in 10 nM Ca^{++}-Ringer's. An aliquot (3–5 μl) of OS-IS from a stock suspension in 1 mM Ca^{++}-Ringer's was placed in the chamber containing 10 nM Ca^{++}-Ringer's. The OS-IS were incubated in the low Ca^{++} for 5 min before recording. The membrane current was measured by drawing the inner segment of an OS-IS into a suction electrode. The suction pipette contained 10 nM Ca^{++}-Ringer's just as in the bath. At the arrow, the perfusate was changed to one containing 20 μM A23187 (0.4% ethanol) in 10 nM Ca^{++}. Photoresponses could be maintained for at least 1 h in this medium. The flash intensity was 2,430 Rh*.

addition of A23187 (arrow, Fig. 5) had little effect on the magnitude of the dark current or its suppression by light. Similar results can be inferred from Wormington and Cone (1978, their Fig. 4) using an ion permeability assay with isolated frog rod outer segments. It seems unlikely that sufficient stores of intracellular Ca^{++} remain through the period shown (20 min) to be a source of the light-stimulated increases in cytoplasmic Ca^{++} that cause suppression of the conductance. In similar experiments, photoresponses to flashes presented every 11 s persisted for at least 1 h. Even if Ca^{++} were released upon illumination, the shunting of much of this Ca^{++} by A23187 to the large Ca^{++}-buffering capacity of the external medium should begin to reduce the magnitude of the photoresponse, an effect that was not observed. Also, the Ca^{++} shunt provided by A23187 would be expected to impair the active reuptake of Ca^{++} released by light.

Experiments with ionomycin, a Ca ionophore having a much greater selectivity for the translocation of Ca^{++} compared with Mg^{++} (Liu and Hermann, 1978; Kauffman et al., 1980), have yielded results very similar to those described for

A23187 in Figs. 5 and 8 (data not shown). To examine the possibility that, in 10 nM free Ca^{++}, A23187 could be transporting Mg^{++} rather than Ca^{++}—the dissociation constants of A23187 for Ca^{++} being 0.4 (Pfeiffer and Lardy, 1976) and 0.2 μM (Wulf and Pohl, 1977)—the Mg^{++} concentration was lowered from the normal 2 mM to 100 μM in several experiments. The addition of A23187, as described for Fig. 5, had no effect on the dark current magnitude or photoresponse.

In addition to not being required for closure of the light-regulated conductance, light-induced changes in Ca^{++} probably do not contribute to shaping the response kinetics. Fig. 6 shows a comparison of the response kinetics before and

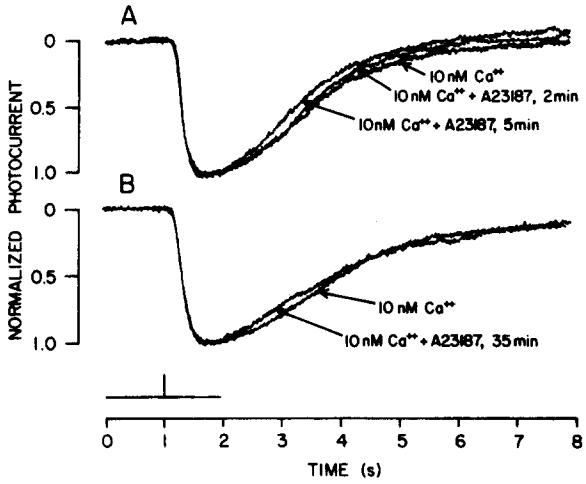

FIGURE 6. Response kinetics are not changed by the addition of A23187 in 10 nM Ca^{++}-Ringer's. In *A*, two digitized photoresponses were averaged, and the averages were normalized to the same peak amplitude. The sampling interval was 10 ms. The protocol was the same as that described in Fig. 5. Flashes were delivered every 11 s for the entire recording period, and the photoresponses obtained 2 and 5 min after changing to a perfusate containing 20 μM A23187 in 10 nM Ca^{++} were digitized and stored. In *B*, under the same recording conditions described for *A*, photoresponses from a different OS-IS were obtained 35 min after the addition of A23187. Under these conditions, there were only small effects on the wave shape. The flash intensity was 830 Rh* in *A* and 2,430 Rh* in *B*.

after the addition of A23187 in 10 nM Ca^{++}-Ringer's. In Fig. 6*A*, there is very little difference between the control photoresponse and the responses obtained 2 and 5 min after the addition of A23187 in 10 nM Ca^{++}-Ringer's. Fig. 6*B* demonstrates similar results for a different OS-IS and a higher light intensity after 35 min in the presence of 10 nM Ca^{++} and A23187. One might anticipate that any component of the waveform resulting from changes in the intracellular concentration of free Ca^{++} would be altered by the presence of A23187, as discussed above. Changes in the waveform kinetics over these long time periods were not observed.

Fig. 7 shows response sensitivity measurements determined from the flash

intensity-response amplitude relation for both 1 mM Ca^{++} and 10 nM Ca^{++}-Ringer's in separate experiments. The OS-IS were desensitized by 1.5 log units in the 10 nM Ca^{++}-Ringer's as compared with 1 mM Ca^{++}, in agreement with others (Yoshikami and Hagins, 1973; Bastian and Fain, 1982, see their Fig. 2*c*). The slight shift of the sensitivity curve to the right after A23187 addition may reflect the time dependence of the desensitization in low Ca^{++}; Bastian and Fain (1982) observed a similar time-dependent shift in the sensitivity between 5 and 10 min in 10 nM Ca^{++}-Ringer's lacking A23187. The small effect of ionophore addition suggests that the Ca^{++} content might have already been reduced by the long incubation with Ca^{++}-Ringer's; this notion is consistent with the results shown in Figs. 1*B*, 2, 5, and 6.

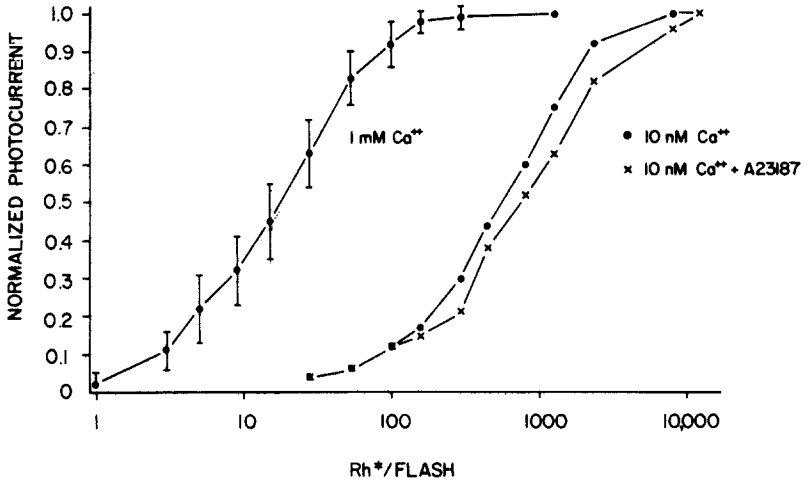

FIGURE 7. Response sensitivity before and after the addition of A23187 in 10 nM Ca^{++}-Ringer's. The data points comprising the flash intensity-response amplitude curve for 1 mM Ca^{++}-Ringer's are the mean ± SD from 24 OS-IS. The protocol for the 10 nM Ca^{++}-Ringer's was the same as that described in Fig. 5. After 5 min in the low Ca^{++}-Ringer's, the flash intensity-response amplitude relation was determined. At this point, the perfusate was changed to one containing 20 μM A23187. After 10 min in the A23187-Ringer's, the intensity-response curve was again determined. The points for the 10 nM Ca^{++}-Ringer's represent the means from four OS-IS before and after the addition of A23187.

Suppression of the Photoresponse by Addition of A23187 in 1 mM Ca^{++} and Its Restoration by Lowering Ca^{++}

The addition of A23187 to an OS-IS in normal Ringer's containing 1 mM Ca^{++} causes suppression of the current flowing in the dark and a loss of the response to light (shown in Fig. 8*A*). Within 15 s after the solution change, a 75% reduction in the photoresponse amplitude was observed; this corresponds to the time course for the release of Ca^{++} shown in Fig. 1*A*. Complete suppression of the light response took ~2 min (see discussion below). Photoresponses that were abolished by A23187 could not be elicited by increasing the flash intensity from

55 to 830 Rh*. Suppression of the photoresponse is consistent with previous studies reporting similar results upon raising extracellular Ca^{++} levels (Yoshikami and Hagins, 1973; Yau et al., 1981; Hodgkin et al., 1984). The results of Fig. 8*A* are consistent with the notion that endogenous Ca^{++} transport systems

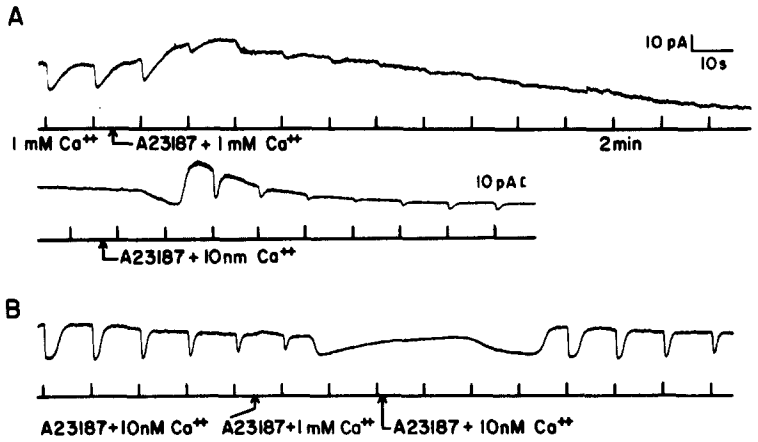

FIGURE 8. Effects of A23187 on the dark current and photoresponse at different Ca^{++} concentrations. Trace *A* shows suppression of the dark current and photoresponse when A23187 is added to OS-IS in 1 mM Ca^{++}-Ringer's. Control photoresponses to 12-ms flashes bleaching 55 Rh* every 11 s were obtained. At the arrow, the normal 1 mM Ca^{++}-Ringer's perfusing the chamber was switched to normal Ringer's containing 20 μM A23187 and 0.4% ethanol. The slow upward drift in the current trace reflects the junctional current from the ethanol in which the ionophore was dissolved. The intensity of the last flash in the top trace was increased to 830 Rh*. In the second trace of *A*, which is continuous with the first (note change in recording gain), the photoresponse could be restored by switching the perfusate to A23187-containing Ringer's with 10 nM Ca^{++}. The flash intensity was 830 Rh*, except for the last two flashes, where the intensity was increased to 2,430 Rh*. Trace *B* shows that the duration of the photoresponse can be modulated by the intracellular Ca^{++} load imposed on the cell by A23187 and the Ringer's solution. A different OS-IS than that shown in *A* has already passed through the high-Ca^{++}, low-Ca^{++}, high-Ca^{++}, low-Ca^{++} cycle in the presence of 20 μM A23187. Note that the first photoresponse in *B* has a duration similar to that observed in the control responses in *A*, but the responses become more rapid as the Ca^{++} depletion continues. The reversibility of Ca^{++} suppression and restoration of the photoresponse was shown again by switching the perfusate to 1 mM Ca^{++} and then back to 10 nM Ca^{++}. The flash intensity was 1,335 Rh*; the current and time scale are the same as in the first trace of *A*.

responsible for maintaining the low levels of intracellular Ca^{++} are not capable of competing with the A23187-induced influx of Ca^{++}.

The second trace in Fig. 8*A* shows that upon lowering the external Ca^{++} concentration to 10 nM in the presence of A23187, the OS-IS light response can be restored. The reduction of the external Ca^{++} resulted in a large transient

increase in the membrane current, current noise (see Matthews, 1985), and the amplitude of the photoresponse. The amplitude of the response relaxed with time to approximately the values observed under normal conditions, as described previously by Hodgkin et al. (1984).

The data of Fig. 8*B* are taken from a separate experiment in which one high-Ca^{++}, low-Ca^{++}, high-Ca^{++}, low-Ca^{++} cycle had been carried out in the presence of A23187. The recording shows more clearly the first and subsequent photoresponses after returning again to 10 nM Ca^{++}-Ringer's, and makes the point that intracellular Ca^{++} levels may influence the mechanism that controls the duration of the light response. Even after the internal Ca^{++} load imposed by A23187 and 1 mM Ca^{++} had been lowered sufficiently to restore light regulation of the current, the response duration was similar to the control responses in 1 mM Ca^{++} (first two responses in Fig. 8*A*). With time, the response duration became shorter and reached a constant value, which suggested that the Ca^{++}-dependent mechanism had come to steady state. When the perfusate was changed to 1 mM Ca^{++}-Ringer's containing A23187 (first arrow, Fig. 8*B*), the current and light response were immediately suppressed by the influx of Ca^{++}, which easily overcame endogenous Ca^{++} translocation. The initial delay of suppression observed in Fig. 8*A* may have resulted from the time necessary for the partitioning of A23187 into the membranes (see discussion below). Once it is present, as in Fig. 8*B*, the rate of raising and lowering the Ca^{++} concentration is limited by the exchange time of the perfusion system, ~10 s. The entire sequence can be repeated by returning to 10 nM Ca^{++} (second arrow, Fig. 8*B*). The fact that photoresponses take some time to recover when the external Ca^{++} concentration is lowered suggests that there is a delay in the response of Ca^{++}-dependent processes to the change in internal Ca^{++} or that internal Ca^{++} must be reduced to some threshold level before permitting light regulation of the current.

Restoration of the Ca^{++}-suppressed Light Response by IBMX

Fig. 9 demonstrates that altering the cyclic GMP metabolism can restore the photoresponse in OS-IS that are unable to regulate their Ca^{++} concentration. The top record shows the suppression of the dark current and photoresponse caused by the addition of A23187 in 1 mM Ca^{++}-Ringer's (arrow), just as in Fig. 8*A*. The subsequent addition of IBMX, a phosphodiesterase inhibitor (arrow, bottom record), restored the ability of the OS-IS to respond to light. The light responses restored by IBMX were larger and much slower than in normal Ringer's solution, and were similar to those previously described for the addition of IBMX in the absence of A23187 (cf. Capovilla et al., 1983). IBMX is known to elevate cyclic GMP levels in rods (Cohen et al., 1978; Woodruff and Fain, 1982). Thus, it appears that increasing the cyclic GMP concentration may restore the mechanisms that cause the light response under conditions where the cell can no longer normally regulate its intracellular Ca^{++} concentration. The total Ca^{++} content of rods under the above conditions was measured using the techniques previously described for the experiments shown in Fig. 3. When OS-IS were incubated in 1 mM Ca^{++}-Ringer's, the total Ca^{++} content was 1.1 ± 0.1 mol Ca^{++}/mol rhodopsin; for 1 mM Ca^{++} plus 20 μM A23187, the intracellular

Ca^{++} content was elevated to 1.6 ± 0.1 mol Ca^{++}/mol rhodopsin; and for 1 mM Ca^{++} plus 20 μM A23187 plus 50 μM IBMX, the Ca^{++} content was 1.6 ± 0.3 mol Ca^{++}/mol rhodopsin (n = 3). This implies that the cyclic GMP pathway is capable of modulating the light-regulated current independently of the intracellular Ca^{++} concentration. Also, these results suggest that IBMX acts by elevating cyclic GMP (thus enhancing the light-regulated conductance) rather than by acting on a mechanism that lowers intracellular Ca^{++} levels.

The addition of A23187 in 1 mM Ca^{++}-Ringer's takes ~2 min to completely suppress the dark current and the photoresponse. This delay may result from at least two factors. First, the time necessary for A23187 to fully partition into the rod membranes. If one takes the rate of Ca^{++} release as an indicator of the partitioning of the ionophore into the rod membranes, then in Fig. 1*A*—where A23187 is applied to a dense suspension of OS-IS—the equilibration time is

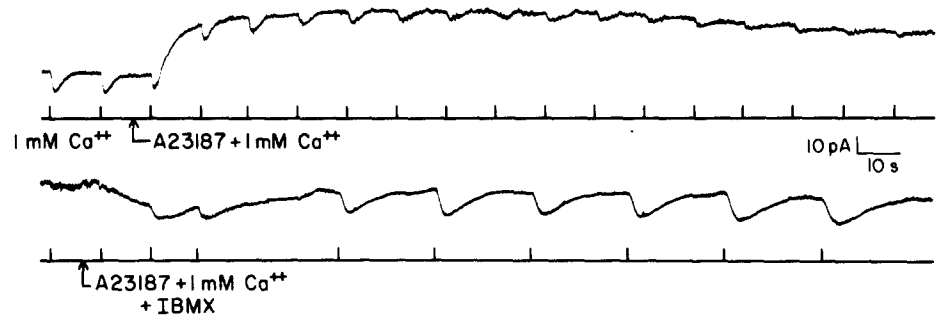

FIGURE 9. Restoration of the photoresponse by IBMX after suppression by A23187 in 1 mM Ca^{++}-Ringer's. The top trace shows suppression of the dark current and photoresponse upon addition of 20 μM A23187 in 1 mM Ca^{++}-Ringer's. Note that the junctional current is larger because of the 2% ethanol compared with 0.4% as shown in Fig. 8*A*. In the second trace, which is continuous with the first, the perfusate was changed (at the arrow) to a Ringer's solution containing A23187, 1 mM Ca^{++}, and 50 μM IBMX. After the addition of IBMX, the photoresponses became larger and much slower than the control responses in the top trace. The flash intensity was 55 Rh*.

rapid and the Ca^{++} release is rapid. However, in Fig. 8*A*, the complete suppression of the dark current and photoresponse resulting from the ionophore-induced influx of Ca^{++} is slow. If the ionophore is given time to partition into the membranes, as in Fig. 8*B*, the elevation of the Ca^{++} concentration causes a rapid suppression of the current. It is important to note that the density of the cells in the two situations (Figs. 1*A* and 8*A*) is very different; the cuvette contains OS-IS that are diluted only 10-fold from a stock suspension, while the electrophysiological recording chamber contains OS-IS that are diluted 70-fold. The probability that the highly lipophilic A23187 will partition into the rod membranes is higher for the denser suspension in the cuvette; this could be reflected in the faster release of Ca^{++} in the spectroscopy experiments. Second, the time dependence of a Ca^{++}-sensitive enzymatic mechanism that regulates the light-regulated channel may be slow (see Figs. 8*B* and 9, effects of IBMX, discussed above). The

experiment also demonstrates that the addition of A23187 in a 1 mM Ca^{++} solution does not uncouple mitochondria in OS-IS, thus abolishing the dark current and suppressing the photoresponse; if this were the case, then one would not expect IBMX to restore the dark current and photoresponse.

DISCUSSION

The central observation of this work is that excitation persists in rod photoreceptors largely depleted of their intracellular Ca^{++} stores. This strongly suggests that changes in the cytoplasmic Ca^{++} concentration are not required for the generation or recovery of the photoresponse. Given the quantitative arguments presented below, it does not seem plausible to propose that a "special" pool of Ca^{++} (<10% of the total) is immune to the action of ionophore and is the source of light-induced Ca^{++} transients. The classic "Ca hypothesis" predicts that photoresponses should progressively deteriorate as Ca^{++} is removed from the cell by repeated light stimulation. This effect is not observed; flashes presented every 11 s elicit normal photoresponses for 1 h in OS-IS that have already lost >90% of their endogenous Ca^{++}. It should be emphasized, however, that the data do not exclude the possibility that Ca^{++}, while not required, may influence the photoresponse observed at physiological levels of extracellular Ca^{++} (see below).

A second major point is that under conditions where endogenous Ca^{++} regulation is overwhelmed (1 mM external Ca^{++} and A23187) and both the dark current and photoresponses are abolished, the subsequent elevation of intracellular cyclic GMP levels by IBMX restores the dark current and its regulation by light. This suggests that changes in the cyclic GMP concentration can modulate the opening and closing of light-regulated channels, independently of internal Ca^{++}. These data are consistent with a model in which cyclic GMP is the primary regulator of the light-regulated channels, with Ca^{++} influencing the levels of cytoplasmic cyclic GMP (cf. Cohen et al., 1978; Kawamura and Bownds, 1981; Woodruff and Fain, 1982; Lolley and Racz, 1982). Thus, the abolition of the dark current observed in 1 mM Ca^{++} and A23187 would reflect closure of the light-regulated channels as cyclic GMP dissociates from the channel because of the decrease in the cyclic GMP content. An enhancement of the dark current observed in low Ca^{++} would result from elevated levels of cyclic GMP, with increased binding of cyclic GMP promoting channel opening. A cyclic GMP–regulated conductance has been demonstrated in the rod plasma membrane (Fesenko et al., 1985; Yau and Nakatani, 1985*b*) and in disk membranes (Caretta, 1985) that is not directly sensitive to or significantly modulated by the Ca^{++} levels in normal ionic media. (The data do not exclude an alternative model in which Ca^{++} and cyclic GMP act on the conductance in parallel pathways with a Ca^{++} change being an obligatory intermediate step before a final unknown transmitter acts on the conductance. No evidence for such a transmitter has yet been obtained.)

Efficacy of the Ca Ionophore

The conclusions of this study rest on our ability to demonstrate that A23187 can alter the Ca^{++} levels in rod photoreceptors, and also on our knowledge of its

speed and site of action. A23187 was used because it selectively exchanges 2 H^+ for 1 Ca^{++} and therefore does not directly disturb the intracellular concentration of monovalent cations or the membrane potential.

Five different observations lead to the conclusion that A23187 distributes into rod photoreceptor membranes and perturbs the intracellular Ca^{++} content. (*a*) More than 50% of the ionophore molecules added to an OS-IS suspension bind to the membranes at a ratio of 6 mol A23187/mol rhodopsin (see Materials and Methods). (*b*) OS-IS incubated with A23187 as observed by light microscopy show a dramatic enhancement of bluish fluorescent that is caused by the accumulation of A23187 (data not shown). (*c*) In 1 mM Ca^{++}-Ringer's, the addition of A23187 abolishes both the rod dark current and its suppression by light, overwhelming the rod's endogenous Ca^{++} transport systems. The light response can be restored either by IBMX or by lowering the extracellular Ca^{++} concentration, which suggests that A23187 does not affect cellular metabolism in a nonspecific or irreversible manner. (*d*) A23187 causes the release of nearly all the Ca^{++} from OS-IS bathed in a K^+ or TMA^+ medium with a low Ca^{++} concentration. (*e*) It is well known that lowering the concentration of extracellular Ca^{++} increases the intracellular cyclic GMP levels by a large amount (see references above). This effect is blocked when OS-IS are isolated and purified in K^+-Ringer's containing 20 nM Ca^{++}. Under these conditions, Na/Ca exchange is inhibited and the stores of intracellular Ca^{++} are retained. However, in an identical OS-IS preparation containing 10 μM A23187, there was a pronounced increase in the cyclic GMP concentration, which indicated that the intracellular Ca^{++} had been released (Cote et al., 1986).

A23187 stimulates either the influx or efflux of Ca^{++}, depending on the direction of the transmembrane Ca^{++} gradient. The size and speed of A23187-induced changes in intracellular Ca^{++} depend on the transport capacity of the ionophore compared with the translocation of Ca^{++} by three possible endogenous transport systems.

(*a*) Ca^{++} flux through the light-regulated channel: A typical value for the dark current in a frog rod is 20 pA. If 3–5% of the dark current can be carried by Ca^{++} (Hodgkin et al., 1984), then this is equivalent to an influx of $2–3 \times 10^6$ Ca^{++}/rod·s.

(*b*) Ca^{++} flux through the Na/Ca exchange mechanism: The maximum transport capacity of the Na/Ca exchanger has been determined for toad and frog rods to be $\sim 10^8$ Ca^{++}/rod·s (Yau and Nakatani, 1984; Schnetkamp, P. P. M., and M. D. Bownds, manuscript in preparation), and for bovine rods to be $\sim 10^7$ Ca^{++}/rod·s (Schnetkamp, 1986). The dissociation constant of the Na/Ca exchange mechanism for Ca^{++} has been determined to be ~ 1 μM (Schnetkamp, 1979, 1980).

(*c*) Ca^{++} flux through a Ca^{++}-ATPase: This is a hypothetical case and there are arguments against the existence of such a mechanism in either the disk or plasma membrane (see Schnetkamp, 1981; Kaupp and Schnetkamp, 1982). The turnover number of the most powerful Ca^{++} pump (found in sarcoplasmic reticulum) is ~200/s (McLennan and Holland, 1975). The most abundant protein in the disk membrane besides rhodopsin is found at ~1 copy/300–1,000 rhodopsins

(rim protein, Papermaster et al., 1978). The maximum number of Ca ions transported, should a Ca^{++} pump exist at this frequency in the disk membrane, would be ~2–6 × 10^5 Ca^{++}/disk·s or 2–6 × 10^8/rod·s.

In contrast, the ionophore A23187 binds to frog rod membranes up to a ratio of 6 mol A23187/mol rhodopsin. Taking the rhodopsin density in the plasma membrane as 3 × $10^4/\mu m^2$ and a surface area for the rod plasma membrane as ~1,100 μm^2, one can estimate the incorporation of ~2 × 10^8 A23187 molecules/rod. The dissociation constant of A23187 for Ca^{++} was determined to be 0.2–0.4 μM (Pfeiffer and Lardy, 1976; Wulf and Pohl, 1977). The maximum turnover number for A23187 is 20/s (Wulf and Pohl, 1977). Thus, A23187 is capable of transporting a maximum of 4 × 10^9 Ca^{++}/rod·s. It is important to note that the dissociation constant of the Na/Ca exchange mechanism is about two- to fivefold higher than the dissociation constant for A23187. When the ionophore is partitioned into the membranes and the Ca^{++} concentration is lowered, all the endogenous Ca^{++} transport systems and the ionophore are exposed to the same Ca^{++} conditions. The efficacy of the Na/Ca exchanger and the ionophore will be equally affected by the lowered Ca^{++} concentration.

It would appear that the maximal Ca^{++} translocation through A23187 is at least 10-fold more potent than endogenous transport systems, which suggests that A23187 can largely determine the intracellular Ca^{++} content.

Possible Roles for Ca^{++}

The results presented in this study, together with the recent observations of Matthews et al. (1985), Yau and Nakatani (1985*a*), and Hodgkin et al. (1984, 1985), make the compelling case that changes in intracellular Ca^{++} levels are not required for the closure of the light-regulated channel. Also, under conditions where the rod has been largely depleted of internal Ca^{++}, the results of Figs. 5 and 6 suggest that channel reopening does not require changes in Ca^{++} content as well. However, raising and lowering the internal Ca^{++} concentration in the presence of A23187 clearly has an effect on the magnitude of the dark current and the photoresponse (Fig. 8).

Several observations suggest that both the waveform and the sensitivity of the photoresponse might be influenced by Ca^{++}. Hodgkin et al. (1984, 1985) have interpreted their results to indicate that the recovery phase of the photoresponse may be limited by the effectiveness with which the Na/Ca exchange functions to remove acquired internal Ca^{++} flowing into the rod through the light-regulated channel. The results of Fig. 8*B* in this study are consistent with a Ca^{++} removal mechanism being rate-limiting in determining the duration of the photoresponse during the recovery from high internal Ca^{++}. Thus, under normal physiological conditions (1 mM external Ca^{++}), it is possible that accumulation of cytoplasmic Ca^{++} in the rod can slow or block recovery of the dark current. All of these points suggest that Ca^{++} plays a modulating rather than a causal role in channel closing and reopening. This is in accord with growing evidence that cellular activation and secretion in other systems can occur in the absence of changes in cytoplasmic Ca^{++} levels (Rink et al., 1983; Pozzan et al., 1983; Michell, 1983; Meldolesi et al., 1984).

Lowering the external Ca^{++} concentration (in the presence or absence of A23187) causes a large decrease in the response sensitivity so that a more intense flash is required to produce a given response amplitude (Yosikami and Hagins, 1973; Bastain and Fain, 1979; this study, Fig. 7). This is a delayed effect and probably results from Ca^{++}-dependent processes modulating the light-adaptational state of the rod. It is noteworthy that desensitization caused by continuous background illumination (Baylor and Hodgkin, 1974; Baylor et al., 1980) has an effect similar to lowering cytoplasmic Ca^{++} in that the recovery phase of the photoresponse to a test flash is accelerated. This is consistent with the suggestion that light causes a decrease in the intracellular Ca^{++} content (Bownds, 1980; Yau and Nakatani, 1985*a*). We are currently evaluating the role of Ca^{++} in desensitization and the recovery phase of the photoresponse by investigating light adaptation in Ca^{++}-depleted OS-IS.

One obvious role for Ca^{++} is to influence the metabolism of cyclic GMP. The enzymes regulating intracellular cyclic GMP levels are known to be Ca^{++} sensitive (phosphodiesterase: Robinson et al., 1980; guanylate cyclase: Lolley and Racz, 1982). The result that Ca^{++} suppression of the dark current and photoresponse can be relieved by the phosphodiesterase inhibitor IBMX (Fig. 9) may be a further indication that the effects of Ca^{++} on the light-regulated conductance are linked to cyclic GMP–related chemistry. The relation between Ca^{++} and cyclic GMP levels is not a simple one (Woodruff and Fain, 1982; Cote et al., 1986), and future work on the efficacy of cytoplasmic Ca^{++} to influence the pathways involving cyclic GMP is needed.

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