Biochimica et Biophysica Acta, 468 (1977) 194—208

BBA 77743

CALCIUM CONTENT OF FROG ROD OUTER SEGMENTS AND DISCS

ETE Z. SZUTS * and RICHARD A. CONE

Thomas C. Jenkins Department of Biophysics, The Johns Hopkins University, Baltimore, Md. 21218 (U.S.A.)

(Received December 7th, 1976)

Summary

To test the "Ca^{2+} hypothesis of visual excitation", we measured the total Ca^{2+} content of freshly isolated bullfrog rod outer segments, and have compared the total Ca^{2+} contents of fully dark-adapted discs with discs exposed to small amounts of light. Discs were prepared by hypotonically lysing outer segments under conditions expected to remove Ca^{2+} from the cytoplasm but not from the discs. Ca^{2+} was assayed by atomic absorption spectrophotometry. We find that both discs and outer segments contain a total of about 0.1—0.2 Ca^{2+} per rhodopsin molecule. Thus, each frog disc retains about $2 \cdot 10^5$ Ca^{2+}. If most of this Ca^{2+} were free in the aqueous space inside the intact discs, the Ca^{2+} activity would be a few mM. Since the light-regulated Na^+ channels have been reported to be highly sensitive to cytoplasmic Ca^{2+}, this store of Ca^{2+} in the discs is far more than required by the Ca^{2+} hypothesis. However, despite several variations in experimental conditions, we did not observe any light-activated release of Ca^{2+} from discs in response to stimuli that photoactivated a small fraction of the rhodopsin, as required by the Ca^{2+} hypothesis. In the 26 experiments reported here we could have detected a release as small as 20—30% of the Ca^{2+} content of the disc.

Introduction

A flash of light transiently hyperpolarizes vertebrate photoreceptors. In rods, this hyperpolarization is initiated by photoactivation of rhodopsin, the visual pigment contained in the disc membranes of the outer segment. Baylor and Fuortes [1] proposed that photoactivated rhodopsin releases from the discs an intracellular substance or transmitter that acts upon the Na^+ channels in the plasma membrane. Recently, Yoshikami and Hagins suggested that the intracellular transmitter is Ca^{2+} [2,3]. According to this "Ca^{2+} hypothesis of visual

* Present address: Marine Biological Laboratory, Woods Hole, Mass., 02543 (U.S.A.).

excitation", dark-adapted rods and cones maintain a low Ca^{2+} activity in the cytoplasm (on the order of 10^{-7} M) and a much higher Ca^{2+} activity inside the discs by actively pumping Ca^{2+} from the cytoplasm into the discs. Photoactivated rhodopsin is postulated to release Ca^{2+} from the disc into the cytoplasm. The released Ca^{2+} would diffuse through the cytoplasm to bind to, and reversibly block, the Na^+ channels of the plasma membrane, thereby causing a transient hyperpolarization, which would decay as the Ca^{2+} was returned to the discs by an active pumping process. We summarize here the results of experiments designed to test the Ca^{2+} hypothesis. We measured the total Ca^{2+} content of intact isolated rod outer segments, and, to check the possibility of light-activated release of Ca^{2+}, we measured and accurately compared the Ca^{2+} content of discs derived from illuminated and non-illuminated rod outer segments [4].

Materials and Methods

Fully dark-adapted bullfrogs (*Rana catesbeiana*) were killed by decapitation and their eyes were enucleated. Retinas were teased away from the eyecup under physiological saline (115 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$ and 10 mM Tris, pH 7.4). The small amounts of pigment epithelium, which occasionally adhered to the retina, were removed. If several retinas were needed for an experiment, the retinas already isolated were stored in a light-tight box while the remaining retinas were being dissected. While two retinas were sufficient per experiment to measure Ca^{2+} in intact outer segments, six retinas were required for the experiments in which Ca^{2+} in discs was measured. The retinas were rinsed for about 5 min in 50 ml of isolation medium. The isolation medium usually contained 115 mM NaCl, 2.5 mM KCl and 10 mM Tris (pH 7.4). When the effect of Ca^{2+} activity on the Ca^{2+} content of the isolated outer segments was to be studied, the Ca^{2+} activity of the isolation medium was varied between experiments by adding various amounts of $CaCl_2$ or by adding 3 mM EDTA instead of $CaCl_2$ (yielding a Ca^{2+} activity of about 10^{-12} M).

The dissection and all the procedures for the preparation of the outer segments or discs were performed either under dim red light (60 W bulb, $\lambda > 680$ nm, whose light indirectly illuminated the working area) or under infrared light. The infrared light source was a flashlight, filtered either through a 3.8 mm thick Corning 2540 glass filter ($\lambda > 880$ nm, Corning Glass Works, N.Y.) or through two 3 mm thick Schott RG1000 filters ($\lambda > 960$ nm, Fish-Shurman Inc., N.Y.). The infrared converter was a headmounted viewer (Find-R-Scope, Model 80300, FJW Industries, Ill.). Both the red and the infrared light sources were calibrated psychophysically: the red bulb was estimated to bleach $\leqslant 4 \cdot 10^{-3}$ %/min; the infrared source was estimated to bleach $\leqslant 4 \cdot 10^{-5}$ %/min with the Corning filter, and $\leqslant 10^{-6}$ %/min with the Schott filters. The total infrared exposure time was not more than 20 min for the outer segments or discs in the experiments of Table II. Thus, when the Schott filters were used in these experiments, less than one rhodopsin in every four discs could have become photoactivated before the time of the light stimulus.

Rod outer segments were isolated by one of three techniques: vortexing, stream-shearing, or shaking the retinas. If they were to be vortexed, one or two

retinas were placed into a test tube, already filled with 10 ml of 10%-Ficoll-containing saline solution per retina. The test tubes were vortexed with a Vortex-Genie (Fisher Scientific Co., Pa.) for 3 to 5 min at the lowest setting. The retinas were then removed, leaving behind a suspension of isolated outer segments. For stream-shearing, a single retina with the receptor side up was placed on a filter paper, and a stream of saline solution that contained 10% Ficoll was directed at it. An infusion pump (Harvard Apparatus, Mass.) delivered the 15 ml solution through a 25-gauge needle at a flow rate of 0.1 ml/s and the runoff with the isolated outer segments was collected. With the third technique, the retinas were placed into a test tube that contained 2 ml of the isolation medium and the rod outer segments were shaken off by agitating the retina with forceps for 2 to 3 min, or by sealing the test tube with Parafilm and shaking the tube for 2 to 3 min. The retinas were then removed from the outer segment suspension. The shaking procedure removed more than half the outer segments from each retina, while the stream-shearing and vortexing procedures removed smaller fractions. The Ca^{2+} contents of the intact rod outer segments did not appear to vary with the technique employed for this isolation step (see Table I). The shaking procedure was the technique used in the experiments of Table II. Following isolation, most of the contaminating tissue fragments were allowed to settle out of the suspension of outer segments (at $1 \times g$ for 1 min for column heights of about 2 cm) and the remaining suspension was filtered through a 37 μm nylon mesh.

Subsequent steps differed depending on whether intact outer segments or discs were to be observed. When Ca^{2+} in the intact outer segments was to be measured, the filtered outer segments were collected into a pellet by centrifuging them at $1900 \times g$ for 5 min. In all but two experiments, the outer segments were collected within 15—20 min of their isolation. In two experiments (Expts. 87 and 88) the outer segments were collected at about 60 min because they were additionally purified on a Ficoll density gradient. In these experiments, 34-ml cellulose tubes were prepared by layering 5 ml of 10% Ficoll saline over 5 ml 25% Ficoll saline solution. The vortexed outer segments, already in a 10% Ficoll suspension, were then placed on top of the column. The tubes were centrifuged in a Beckman SW 25.1 rotor in a Beckman Model L centrifuge for 5 min at $58\,000 \times g$. The carpet of outer segments between the 10% and 25% Ficoll layers was removed in about 2 ml volume and was washed with 18 ml of saline. The outer segments were pelleted by centrifuging for 4 min at $1900 \times g$. The supernatants were discarded and replaced with fresh 10 ml saline before the resuspensed outer segments were collected with a final spin at $1900 \times g$.

To measure the Ca^{2+} content of rod discs and to attempt to detect light-regulated Ca^{2+} release from discs, the procedure outlined in Fig. 1 was performed on isolated outer segments. In these experiments, the initial outer segment suspension was divided into two equal aliquots. The aliquots were simultaneously withdrawn from a well-stirred suspension of outer segments with the aid of a specially designed mechanical device using identical pipettes, so that an equal division of both outer segments and contaminating debris could be accomplished. The aliquots were then subsequently handled as identically as possible, except for the exposure of the test aliquot to a light flash. The test tubes containing the aliquots were placed into two separate light-tight boxes

and the test aliquot was illuminated with a light flash from an M3 flash unit (Honeywell, Minn.) that has about a 30 ms flash duration. A 2 mm thick Schott KG3 infrared-absorbing filter, a Wratten No. 25 filter (Eastman Kodak Co., N.Y.) and a sheet of white diffusing paper were placed in the light path to provide diffuse light at 620 nm. With such illumination, light scatter by the organelles and self-screening by rhodopsin were minimized. The flash from a single M3 bulb bleached 5.2 ± 0.5% of the rhodopsin and to decrease this bleaching intensity, calibrated Wratten No. 96 neutral density filters were placed in the light path. Immediately following the light flash, the outer segments in both aliquots were hypotonically shocked by vigorously squirting 10 ml of hypotonic solution into both test tubes. After mixing, the final tonicity of the solution was about 0.1 × isotonic. The shocking solution contained 3 mM EDTA to chelate the available cytoplasmic Ca^{2+} and to remove it into the suspending solution. Thus as a result of the shock, the discs were exposed to a solution with a Ca^{2+} activity of $<10^{-9}$ M. Immediately after delivering the shock, the suspension of lysed outer segments was centrifuged; the pellet to be analyzed was formed within 5 min of the shock. In most of these experiments, an isotonic solution containing 120 mM choline chloride and 10 mM Tris (pH 7.4) was used for the isolation medium to permit simultaneous measurements of the Na^+, K^+, Mg^{2+} and Ca^{2+} contents of discs. (The results of some of the Na^+, K^+ and Mg^{2+} measurements are briefly described elsewhere [4]). The composition of the isolation media in Expts. 89, 98 and 99 was the usual 115 mM NaCl, 2.5 mM KCl and 10 mM Tris (pH 7.4). The experiments of Fig. 1 were designed to minimize the time intervals between the isolation of the outer segments, the delivery of the light stimulus, and the collection of the sample into a pellet for Ca^{2+} analysis. As a result, less than 15 min elapsed following the isolation of the outer segments before they were exposed to the test flash and the hypotonic shock. Thus, the outer segments were tested for light-activated Ca^{2+} release when they were still fresh and mostly intact (see below).

Light-activated Ca^{2+} release in intact photoreceptors was studied in six experiments (Expts. 69, 71, 73, 74, 93 and 94) by hypotonically shocking excised retinas. A pair of retinas were dissected under infrared illumination and were soaked for 5 min in 50 ml of saline solution that contained no $CaCl_2$ and to which 3 mM EDTA was sometimes added. The retinas were then removed and placed into separate containers. A calibrated light flash from an M3 flash unit was applied to the test retina about one second before delivering the hypotonic shock solution (containing 3 mM EDTA) to both test and control retinas. The vigorously delivered ten-fold hypotonic shock caused most of the outer segments to break off the retina and to fragment. The bulk of the retinal tissue was not dispersed by the shock and was easily removed, leaving behind a suspension of osmotically shocked outer segments and shocked cellular contaminants. The suspensions were centrifuged and the Ca^{2+} contents of the pellets and supernatants were analyzed with atomic absorption.

All the procedures subsequent to the final sedimentation of the intact or lysed outer segments were identical. The supernatants were removed and saved, leaving small pellets of intact or lysed outer segments (with adhering supernatant) in the test tubes. The total volume of outer segments or discs in the pellet was never more than a few percent of the volume of adhering super-

natant. A measured amount of 25% emulphogene BC-720 solution was added to a final 2% concentration to solubilize the pellets. The volumes of the "solubilized pellets" (50—100 μl) were determined by weighing, and a measured aliquot (10—30 μl) was taken from each for rhodopsin analysis. Rhodopsin concentration was determined in a Cary 17 spectrophotometer (Cary Instruments, Cal.) by measuring the difference spectrum of rhodopsin before and after bleaching in the presence of 2 mM NH_2OH, and by using 42 000 $(M \cdot cm)^{-1}$ for the molar extinction coefficient at 500 nm [5]. The Ca^{2+} contents of the remaining "solubilized pellets" and of the corresponding supernatants were measured with a flameless atomic absorption spectrophotometer (Model 61 or 63, Varian Techtron, Ca.). Atomic absorption spectrophotometry measures the total concentration of ions and not their activity. Tests for both chemical and nonatomic interferences were performed and both of these interferences were found to be absent during the spectrophotometric analysis of Ca^{2+} and Mg^{2+}. Ca^{2+} or Mg^{2+} concentrations in each of the "solubilized pellets" and supernatants were determined by comparing the signals of three or more 1 μl aliquots from the solution being analyzed with three or more 1 μl aliquots from an appropriate standard solution. From these samplings, the average Ca^{2+} (or Mg^{2+}) concentration and its standard deviation were calculated for each of the "solubilized pellets" and supernatants. A detailed description of the operation of the instrument and the presentation of experimental data are given elsewhere [4].

Calcium in the "solubilized pellets" could originate from four sources: from the outer segments or discs, from the supernatants that adhered to the pellets, from the added detergent solutions, and from possible contamination introduced during handling. Therefore, the relationship for calculating the Ca^{2+} content of outer segments or discs is

$$Ca_t V_t = X + Ca_s V_s + Ca_d V_d + Ca_c$$

where

Ca_t = total Ca^{2+} concentration of "solubilized pellet", measured by atomic absorption

V_t = volume of "solubilized pellet", determined by weighing

X = moles of Ca^{2+} from rod outer segments or discs

Ca_s = total Ca^{2+} concentration of supernatant, measured by atomic absorption

V_s = volume of the supernatant that adhered to the pellet. V_s was taken to be equal to the volume of the pellet, which was determined by weighing. The error introduced by such an approximation was negligible, because the pellet volume was almost entirely composed of supernatant and because the total Ca^{2+} concentration in the supernatant was much lower than in the "solubilized pellet".

Ca_d = total Ca^{2+} concentration of detergent, measured by atomic absorption

V_d = known volume of detergent added to pellet

Ca_c = moles of Ca^{2+} from handling, as measured in control blanks subjected to the same procedures as the sample.

The Ca^{2+} contributions from handling (Ca_c) and from the added detergent solution ($Ca_d V_d$) were often measured and were usually found to be negligible in

most experiments, since their combined contribution was usually less than the standard deviation in Ca_tV_t. In any given experiment the standard deviation of Ca_tV_t was usually between 5—15%. The Ca^{2+} associated with the outer segments or discs, as derived from the above equation, was then normalized to the rhodopsin content of the pellet and was expressed as Ca^{2+} per rhodopsin molecule, Ca^{2+}/rho. The standard deviations in the calculated Ca^{2+}/rho were set primarily by the reproducibility of the atomic absorption measurements. As will be shown later, the variability in results between experiments exceeded the measuring errors within any given experiment. When ions other than Ca^{2+} were to be measured in the outer segments, calculations analogous to the above were performed.

The degree to which the isolation procedures and the different handling procedures damaged the plasma membrane of the outer segments was monitored by observing the extent to which didansylcystine stained outer segments [6] or the extent to which osmotic shocks affected the volumes of the organelles. All three of the techniques used for the isolation of the outer segments were found to cause only 20—30% of the outer segments to be leaky to didansylcystine. The fraction of leaky outer segments did not increase with further handling and with the subsequent sedimentation of the outer segments into a pellet at 1900 $\times g$. However, resuspending the pellets did cause further damage. Thus, when outer segment suspensions were subjected to the same procedure outlined in Fig. 1, about 40—50% of the outer segments were leaky to didansylcystine at the time the light flash was delivered. A similar fraction of the outer segments (30—50%) was found to possess damaged plasma membranes when the osmotic responsiveness of the outer segments was tested.

All solutions were prepared in distilled, deionized water with reagent grade chemicals from Baker Chemical Co., N.J. Tris, choline chloride and the free acid of EDTA were purchased from Sigma Chemical Co., Mo. The Ca^{2+} impurity in these chemicals contributed less than 10^{-7} M to the total Ca^{2+} concentration of the final solutions. As supplied, emulphogene BC-720 (GAF Corp., N.Y.) and Ficoll 400 (Pharmacia Fine Chemicals, N.J.) were seriously contaminated with Ca^{2+} and other ions. To remove Ca^{2+}, aqueous 25% emulphogene was passed through a mixed-bed ion exchanger (Illinois Water Treatments Co., Ill.). Ficoll was dissolved in a solution of 3 mM EDTA, 100 mM NaCl (Tris-buffered at pH 8) and then dialyzed against 100 mM NaCl to remove chelated Ca^{2+} and uncomplexed EDTA. NaCl was later removed by dialysis against deionized water. Arsenazo III was purchased from Aldrich Chemical Co., Wisc. Contaminating Ca^{2+} was removed by passing a 1 mM stock solution (adjusted to pH 7.5 with NaOH) through a column of Chelex 100 (Na form, 100—200 Mesh, Bio-Rad Laboratory, Ca.).

All experiments were performed at room temperature.

Results and Discussion

The Ca^{2+} content of isolated rod outer segments was observed in solutions with a wide range of Ca^{2+} activity and with several variations in procedure, as described in Table I. Despite these variations, in all 6 experiments, the outer segments contained between0.1 and 0.6 Ca^{2+}/rho. The experiments were

TABLE I

EXPERIMENTAL DATA USED FOR THE DETERMINATION OF THE Ca^{2+} CONTENT IN INTACT ISOLATED ROD OUTER SEGMENTS

The Ca^{2+} content of the outer segments was calculated from these experimentally determined parameters, using the equation presented in Materials and Methods. Data not tabulated here (e.g. Ca^{2+} contribution of detergent solution) are presented elsewhere [4]. In Expts. 79, 87, 88 and 109, all the steps up to the separation of supernatant from the final pellet were performed under infrared illumination. Dim red light was used in the other experiments. The outer segments were isolated by the shaking procedure in Expts. 79, 109 and 116, by the vortexing procedure in Expts. 87 and 88, and by the stream-shearing procedure in Expts. 105 and 107. As shown in the second column, the Ca^{2+} activity of the washing and suspending solutions was varied between experiments to determine whether this affected the Ca^{2+} content of the isolated organelle. The isolated outer segments were collected within 15—20 min of their isolation, except in Expts. 87 and 88 where they were collected at about 60 min because of an additional density gradient centrifugation. The procedures for isolating the outer segments caused 20—30% of the organelles to become leaky to didansylcystine. The same test was also performed on aliquots that were set aside during Expts. 107, 109 and 116, and less than 30% of the outer segments were leaky even though 100 min elapsed between their isolation and the test. In some experiments, the outer segment suspension was divided into two aliquots following filtration and the Ca^{2+} content of the outer segments in each aliquot was measured. The difference between such duplicates probably reflects variable contributions from contaminating cells. The high Ca^{2+} content in Expt. 109 correlated with unusual vesicular contamination in the outer segment suspension, as observed with the light microscope. In Expt. 105 only an upper bound could be obtained because the relatively high Ca^{2+} in the suspending solution masked Ca^{2+} from the outer segments. In Expt. 116, the Ca^{2+} liberated upon solubilizing the pellet was measured with 30 μM arsenazo III using the following parameters for the calculations: $K = 10^{-5}$ M and $\Delta\epsilon_{650} = 18.5 \cdot 10^3$ $(M \cdot cm)^{-1}$.

Expt.	Ca^{2+} activity in suspending solution	Total Ca^{2+} concn. in solubilized pellet	Volume of solubilized pellet	Total Ca^{2+} concn. in supernatant	Pellet volume (mainly, adhering supernatant)	Rho content of solubilized pellet	Ca^{2+} content
	(M)	(μM) *	(μl)	(μM) *	(μl)	(nmol) **	(Ca^{2+}/rho) *
79	$<10^{-9}$	41.5 ± 1.4	110	11.9 ± 2.7	60	9.6	0.40 ± 0.02
		28.8 ± 1.0	120	9.5 ± 0.7	70	8.1	0.34 ± 0.02
87	$<10^{-9}$	4.2 ± 0.2	123	1.1 ± 0.1	102	2.7	0.15 ± 0.01
		4.0 ± 0.3	108	1.0 ± 0.03	86	2.6	0.13 ± 0.01
88	$<10^{-9}$	11.9 ± 0.4	67	1.0 ± 0.08	48	4.1	0.19 ± 0.01
		13.2 ± 0.3	93	0.9 ± 0.05	73	4.1	0.28 ± 0.01
105	10^{-4}	83 ± 12	69	84 ± 12	55	9.2	$\leqslant$0.25
107	10^{-5}	5.5 ± 1.2	57	11 ± 3	9	2.1	0.10 ± 0.04
109	10^{-5}	25 ± 6	84	9.2 ± 1.4	26	3.1	0.61 ± 0.16
116 (arsenazo III)	10^{-6}	3.91	130	1.53	30	2.5	0.11
		3.96	140	1.45	40	2.5	0.12

* Expressed as average ± S.D. In all cases n = 3—5 and is the number of determinations performed with the atomic absorption spectrophotometer.

** There are 10—15 nmol rho per bullfrog retina.

arranged to check whether the plasma membrane of the isolated outer segment was leaky to Ca^{2+} and to determine whether there was significant contamination from other cells or organelles.

The Ca^{2+} permeability of the outer segment plasma membrane can be artificially increased with Ca^{2+} ionophores. In the presence of such ionophores, extracellular Ca^{2+} activities greater than 10^{-6} M substantially reduce the maximal photovoltage responses of rat retinas [7], and decrease the Na^+ permeability of the plasma membrane of isolated outer segments [8], suggesting that

Ca^{2+} enters the outer segment when extracellular $[Ca^{2+}] > 10^{-6}$ M. Correspondingly in the presence of the same ionophores, extracellular Ca^{2+} activities less than 10^{-8} M appear to slowly remove Ca^{2+} from the outer segments. If the plasma membrane of the isolated outer segment became leaky to Ca^{2+} under our conditions, the Ca^{2+} content of the organelles would be expected to increase or diminish as a function of extracellular Ca^{2+} activity. However, we did not detect any such variation. In Expts. 105, 107 and 109 (Table I) the Ca^{2+} activity of the suspending media was kept at 10^{-4} or 10^{-5} M, clearly favorable for Ca^{2+} accumulation by the outer segments. Yet the Ca^{2+} content of the outer segments in these experiments did not differ significantly from the contents found in Expts. 79, 87 and 88, where a Ca^{2+} activity of less than 10^{-9} M in the suspending media would have favored Ca^{2+} losses. These observations suggest that the plasma membrane was essentially impermeant to Ca^{2+}. (In contrast to our finding of an essentially constant Ca^{2+} content in intact outer segments, the Ca^{2+} content of fragmented outer segments has been reported to depend significantly on the Ca^{2+} activity of the suspending solution [9].)

When outer segments are isolated in large quantities, contamination by other cells and organelles is unavoidable, and should increase the apparent Ca^{2+} content of the outer segment. We determined the maximum possible contamination by measuring the Ca^{2+} content of both the excised retina and the pigment epithelium. Bullfrog retinas that were rinsed with the EDTA-containing saline and then solubilized, contained 10 ± 4 Ca^{2+}/rho (S.D. for 6 retinas), which corresponds to about 0.13 μmol Ca^{2+}/retina given an average rhodopsin content of 13 nmoles per bullfrog retina. After similar handling, the entire pigment epithelium was found to contain about 0.45 μmol Ca^{2+} (2 measurements). Since the pellets of outer segments contain two orders of magnitude less Ca^{2+}/rho, it is clear that significant amounts of Ca^{2+} contamination from the retina or pigment epithelium could occur despite the precautions used in the isolation procedure. The contribution of such contaminants was estimated by observing the final Ca^{2+} contents either after additional purification of the outer segments on a Ficoll density gradient (Expts. 87 and 88, Table I) or after hypotonically shocking the outer segment suspension (see below). However, neither of these additional purification procedures significantly reduced the measured Ca^{2+} content. Thus, in the majority of the experiments most of the Ca^{2+} in the pellets probably originated from the outer segments. Therefore, we conclude that the Ca^{2+} content of the outer segments is, to within a factor of two, 0.2 Ca^{2+}/rho. Moreover, we believe this represents the Ca^{2+} content of the outer segments in vivo, since we did not detect any significant loss or gain of Ca^{2+} by the outer segments after they were isolated.

To corroborate the accuracy of the above atomic absorption measurements, further experiments were performed with arsenazo III. Arsenazo III is a Ca^{2+}-chelating dye whose absorption spectrum varies with the Ca^{2+} activity in solution [10]. When pellets of outer segments were solubilized with emulphogene in the presence of arsenazo III, the outer segments were found to liberate about 0.1 Ca^{2+}/rho (Expt. 116, Table I). If endogenous Ca^{2+}-chelating sites were present in the pellets, they would have competed with the dye for Ca^{2+} so that arsenazo III could have detected only a fraction of the total Ca^{2+} present. The effect of such a competition was calculated from the published data on Ca^{2+}

binding by rod outer segments. Hemminki [11] and Weller et al. [12] have found that at a Ca^{2+} activity of 10^{-6} M (the value observed in the solubilized pellets of Expt. 116), homogenized cattle rod outer segments bind 0.2—1.0 μmol Ca^{2+}/g protein. These binding ratios are equivalent to 0.02—0.1 Ca^{2+}/rho (9 nmol of rhodopsin is equivalent to about 1 mg bovine serum albumin by the Lowry assay). Thus, the effect of Ca^{2+} binding is to increase the observed Ca^{2+} content of the outer segments from 0.1 Ca^{2+}/rho to perhaps as much as 0.2 Ca^{2+}/rho. Therefore, the Ca^{2+} content measured by the arsenazo III technique is in good agreement with the Ca^{2+} content of about 0.2 Ca^{2+}/rho observed with atomic absorption.

Since rhodopsin concentration in frog rod outer segments is about 3 mM [13,14], a content of 0.2 Ca^{2+}/rho in the outer segment is equivalent to a total Ca^{2+} concentration of 0.6 mM. Rhodopsin constitutes about 80% of all protein in the outer segment [15] and frog rhodopsin has a molecular weight of about 40 000 [15]. So a content of 0.2 Ca^{2+}/rho is also equivalent to about 5 μmol of Ca^{2+}/g protein. Outer segments thus seem to contain less Ca^{2+}/g protein than either unloaded mitochondria or unloaded sarcoplasmic reticulum vesicles, both of which retain 10—15 μmol Ca^{2+}/g protein [16,17].

Two other reports have been published on the Ca^{2+} content of intact outer segments. The Ca^{2+} content we observe for the isolated outer segment is in reasonable agreement with the findings of Hagins and Yoshikami, who used electron microprobe analysis on quick-frozen and cleaved *Rana pipiens* retinas. They found that total Ca^{2+} in rod outer segments does not exceed 3.8 mM [18], when the extracellular solution contains 0.1 mM Ca^{2+}. On the basis of further work with the electron microprobe, they recently concluded that total Ca^{2+} is about 2 mM [19], which corresponds to about 0.7 Ca^{2+}/rho, a value which appears to agree with ours within experimental uncertainties. In contrast, our findings contradict the results of Hendriks et al. [20] who measured 11 Ca^{2+}/rho in the outer segments of *Rana esculenta*. Their experimental procedure was similar to ours in that they washed the isolated outer segments in solutions of low Ca^{2+} concentration, sedimented the organelles into a pellet, and measured the Ca^{2+} concentration of both the pellets and supernatants with atomic absorption spectrophotometry. It is unlikely that their value of 11 Ca^{2+}/rho is due to species variations, since in a few experiments on *Rana pipiens* and on *Bufo marinus* we again found Ca^{2+} contents of about 0.2 Ca^{2+}/rho. In agreement with our findings, they observed that decreasing the Ca^{2+} activity of the washing solutions with 10 mM EDTA did not significantly reduce the Ca^{2+} content of the organelles.

In several experiments (including Expt. 105, Table I) we measured both the Ca^{2+} and Mg^{2+} content of intact outer segments, and found a Mg^{2+} content of about 1.2 Mg^{2+}/rho. This Mg^{2+} content for intact *Rana catesbeiana* outer segments is also in disagreement with the value of 15 Mg^{2+}/rho reported by Hendriks et al. on *Rana esculenta* [20]. The origin of these orders-of-magnitude higher Ca^{2+} and Mg^{2+} observations by Hendriks et al. is obscure.

To measure the Ca^{2+} content of rod discs and to attempt to detect light-activated Ca^{2+} release from discs, outer segments were hypotonically shocked to remove Ca^{2+} from the cytoplasm but not the Ca^{2+} retained by the discs (see procedure in Fig. 1). The hypotonic shock (0.1 × isotonic) together with the

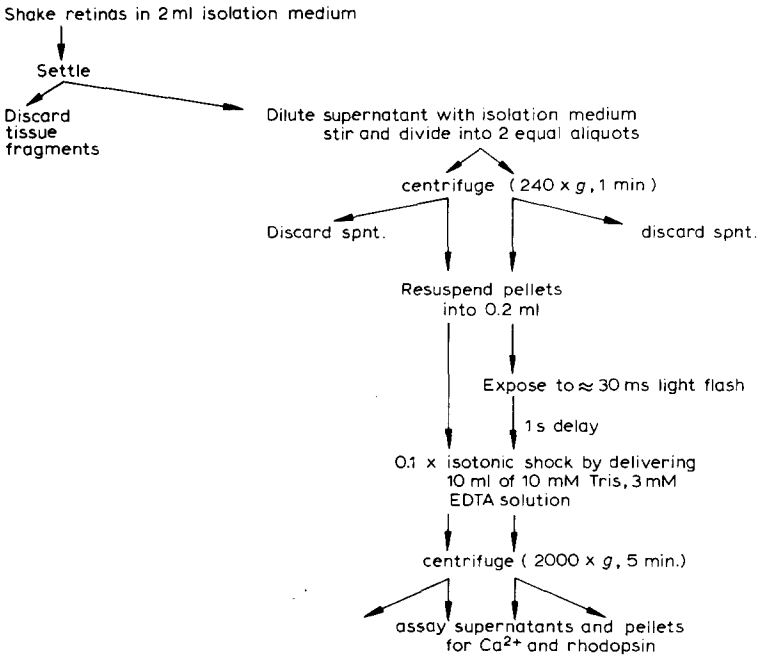

Fig. 1. Flow chart showing the procedure employed for collecting lysed outer segments to measure the Ca^{2+} content of rod discs and possible light-activated Ca^{2+} release from discs. The outer segments were hypotonically shocked within 15 min of their isolation, at which time 50% or more of the outer segments were intact as determined by their osmotic responsiveness and by their staining with didansylcystine.

vigorous delivery of the shocking solution was sufficient to lyse the plasma membrane, because the rod outer segments fragmented into micron-sized particles and because about 90% of the initial Na^+ and K^+ content of the outer segment was found to have been released as measured by atomic absorption. When washed in the isotonic choline solution, both the Na^+ and K^+ contents of the organelles were about 20 ions/rho, while only about 2 ions/rho remained following the hypotonic shock. In contrast, the shock did not appear to have lysed the discs, since the lysed outer segments still retained some Na^+ and K^+. Moreover, they retained a large fraction of the initial Ca^{2+} content of the intact outer segment (see below). Given the flattened shape of the discs, it seems reasonable that the shock did not lyse the discs. Discs have been shown to be good osmometers, at least for small osmotic shocks [21]. If they remain osmometers to 0.1 X isotonicity, the average height of the aqueous space between the membranes of a disc should uniformly increase from about 2 nm [21,22] to about 20 nm. Since the discs are more than 6000 nm in diameter, such a small increase in the distance between the membranes could easily occur without membrane stretching. To see whether discs show morphological evidence of lysis, discs were freeze-fractured after the 0.1 X isotonic shock and examined in the electron microscope. It was found that the discs did not swell uniformly but rather assumed tubular shapes in regions away from the disc rims (Oleszko-Szuts, S., unpublished observations). Such behavior has been previously reported by Falk and Fatt [23] in observations made on fixed and embedded samples. Analysis of the magnitude of increase in volume enclosed by each

freeze-fractured disc indicates that the discs retain all, or at least a major fraction, of their osmotic contents. Thus, the discs did not appear to have lysed. Although the freeze-fracture studies cannot measure Ca^{2+} leakage, they provide additional evidence that suggests that the osmotic shock may not have released Ca^{2+} from the discs. An advantage of a ten-fold hypotonic shock is that it should lyse virtually all contaminating cells and cell organelles other than discs, and thereby greatly reduce Ca^{2+} contamination in the pellet: to survive such a shock unlysed, a cell or an organelle would initially have to be "collapsed", with a very high surface to volume ratio, much like that of a disc. Few, if any, such cells or organelles occur in the retina.

The results of the hypotonic shock experiments are tabulated in Table II. The Ca^{2+} content of the lysed outer segments was found to be 0.05 to 0.36 Ca^{2+}/rho, with a mean of 0.13 Ca^{2+}/rho (20 experiments). Although this value is about 2/3 the amount observed in unshocked outer segments (0.2 Ca^{2+}/rho), the error limits of each of the two types of experiments are such that the difference is not significant. That is, no significant loss of Ca^{2+} appears to have

TABLE II

Ca^{2+} CONTENT OF DISCS AND THE EFFECT OF LIGHT ON THE Ca^{2+} CONTENT

The Ca^{2+} content of discs was calculated from experimentally determined parameters, using the equation presented in Materials and Methods. Only the final results are tabulated here. Experimental data are presented elsewhere [4]. Expts. 47 and 48 were performed under dim red light. From Expt. 49 on, infrared illumination was used for isolating the outer segments and for the subsequent steps up to the final collection of the lysed outer segments. Starting with Expt. 65, even the dissections were performed with infrared illumination.

Expt.	Ca^{2+} content of unilluminated discs in Ca^{2+}/rho *	Ca^{2+} content of illuminated discs in Ca^{2+}/rho *	Percent difference *	No. of photoactivated rhodopsin molecules per disc by the light flash **
47	0.096 ± 0.005	0.049 ± 0.003	49 ± 7	$2 \cdot 10^4$
48	0.054 ± 0.008	0.031 ± 0.007	43 ± 21	10^2
49	0.044 ± 0.014	0.009 ± 0.012	80 ± 49	10
50	0.091 ± 0.008	0.080 ± 0.006	12 ± 11	$2 \cdot 10^{-3}$
52	0.24 ± 0.02	0.11 ± 0.03	54 ± 16	10^4
53	0.18 ± 0.01	0.08 ± 0.01	56 ± 6	10^4
54	0.048 ± 0.011	0.056 ± 0.01	−17 ± 31	20
55	0.075 ± 0.02	0.079 ± 0.02	−5 ± 38	20
56	0.072 ± 0.015	0.062 ± 0.014	14 ± 29	20
57	0.091 ± 0.014	0.071 ± 0.01	22 ± 19	20
58	0.119 ± 0.009	0.103 ± 0.007	13 ± 10	$2 \cdot 10^2$
59	0.15 ± 0.05	0.16 ± 0.05	−2 ± 46	$2 \cdot 10^3$
60	0.093 ± 0.03	0.076 ± 0.009	18 ± 34	$2 \cdot 10^4$
61	0.049 ± 0.002	0.07 ± 0.009	−43 ± 19	$2 \cdot 10^4$
65	0.14 ± 0.01	0.14 ± 0.01	0	20
66	0.36 ± 0.02	0.36 ± 0.02	0	$2 \cdot 10^2$
76	0.22 ± 0.07	0.25 ± 0.07	−14 ± 45	$2 \cdot 10^2$
89	0.085 ± 0.005	0.107 ± 0.006	−26 ± 9	$2 \cdot 10^4$
98	0.18 ± 0.03	0.20 ± 0.03	−11 ± 24	$2 \cdot 10^3$
99	0.18 ± 0.01	0.18 ± 0.01	0	$2 \cdot 10^3$
Average for column ± S.D. $n = 20$	0.13 ± 0.08	0.12 ± 0.09	12 ± 31	

* Expressed as average ± S.D. In all cases, $n = 3$—5 and is the number of determinations performed with the atomic absorption spectrophotometer.

** There are $2 \cdot 10^6$ rho/frog disc.

occurred on hypotonically shocking the outer segments. Since each bullfrog disc contains about $2 \cdot 10^6$ rhodopsin molecules, a mean of 0.1 Ca^{2+}/rho is equivalent to $2 \cdot 10^5$ Ca^{2+} per disc. Most of this Ca^{2+} probably resides inside the discs, unless the exposed membrane surfaces of the lysed outer segments contain unusually strong Ca^{2+} chelating sites. To bind significant amounts of Ca^{2+} under the condition of the experiments (suspending solutions contained 3 mM EDTA and 1—10 μM total Ca^{2+}), the affinity of such chelating sites for Ca^{2+} would have to be greater than about 10^{11} M^{-1} if it is assumed that during the process of lysis, equilibrium is established between the binding sites and Ca^{2+} in solution. If the 0.1 Ca^{2+}/rho were distributed entirely within the aqueous volume of a disc (a space about 2 nm high in vivo), total intradiscal Ca^{2+} concentration would be about 5 mM. Structural analogy between rods and cones suggests that much of this Ca^{2+} may be free, because extracellular Ca^{2+} activity is about 1 mM and the space within the discs of cones is continuous with the extracellular space. However, it is not yet known what fraction of the Ca^{2+} observed in these experiments, if any, exists free inside the rod disc.

Also employing atomic absorption techniques, Liebman [9] measured 4 μmol Ca^{2+}/g protein (equivalent to about 0.2 Ca^{2+}/rho) in the fragmented outer segments of *Rana pipiens*, when he maintained Ca^{2+} activity at less than 10^{-9} M in the bathing solutions. This was the same range of Ca^{2+} activity to which we exposed the discs following the hypotonic shock. Since it is likely that only the plasma membrane was damaged in his fragmented outer segments, Liebman may in effect have measured Ca^{2+} in discs. His value of 0.2 Ca^{2+}/rho is in good agreement with our findings of 0.1 Ca^{2+}/rho. In contrast, Hendriks et al. [20] observed a Ca^{2+} content equivalent to about 6 Ca^{2+}/rho in the discs of hypotonically shocked rod outer segments from *Rana esculenta*. Thus, their observations on both rod outer segments and discs exceed our observations and those of Yoshikami and Hagins [19] and Liebman [9], by nearly two orders of magnitude.

Based on the sensitivity of the Na^+ channels to the cytoplasmic Ca^{2+} activity [7,8,24], the Ca^{2+} hypothesis predicts that at threshold at least 10^2—10^3 Ca^{2+} must be released by a flash that photoactivates one rhodopsin molecule in a frog disc. Thus, each disc must contain at least 10^2—10^3 releasable Ca^{2+} ions. Since the total Ca^{2+} in a disc is many times greater than this, only a small fraction of the total would have to be released to satisfy the Ca^{2+} hypothesis.

Light-activated Ca^{2+} release from discs was calculated for each of the experiments in Table II by comparing the Ca^{2+} contents of the lysed outer segments in control and test aliquots. Great care was taken to assure that both aliquots were handled identically, except for the exposure to light, to make detectable the smallest possible light-activated release. In several of our earlier experiments, differences in Ca^{2+} content between illuminated and non-illuminated discs were observed that significantly exceeded the measurement errors. Although such differences sometimes reflected Ca^{2+} release, the measured light-correlated Ca^{2+} changes varied between various degrees of Ca^{2+} release and uptake (see Table II). Despite numerous repetitions and modifications of the experiment, this variability could not be reduced. Thus on the average, light-activated Ca^{2+} release was not demonstrated. Experiments 47 through 60 in Table II are the same experiments which we previously reported [25]. We then

interpreted these results as an indication of Ca^{2+} release, because they show an overall trend toward release by light. However, this trend was not confirmed in subsequent experiments. Control experiments clearly indicated that the variability in the light-correlated Ca^{2+} changes was not caused by inaccuracies in the atomic absorption measurements. Even though our procedure was designed to minimize it, the unequal division of residual contamination by non-outer segment debris between test and control aliquots may have been the cause of the observed variability between experiments.

Korenbrot and Cone [26] have shown that the phototransduction mechanism remains intact in freshly isolated outer segments when handled by the procedures they describe. In addition, under conditions more similar to those used here, Bownds and Brodie [27] have observed that phototransduction can persist for several hours in the isolated outer segment. Nevertheless, the photosensitivity of the Ca^{2+} release process may have been diminished by our experimental procedures, just as the Na^+ permeability of the isolated outer segments can be drastically reduced under certain circumstances [21,28]. Therefore, we devised a new procedure to attempt to detect light-activated Ca^{2+} release in intact photoreceptors. To accomplish this, rod outer segments were hypotonically shocked with an EDTA-containing solution when they were still attached to the retina. Since the retinas were freshly dissected under dim infrared illumination and handled gently to avoid breaking off the outer segments, the outer segments were expected to be in good physiological condition at the time of the light flash. Since the hypotonic shock was about 0.1 × isotonic, the shock was probably sufficient to lyse and release the Ca^{2+} content of the contaminating cells without releasing the Ca^{2+} content of discs, as discussed above. By this procedure, the Ca^{2+} content of the lysed outer segments was found to be 0.05—0.48 Ca^{2+}/rho, with a mean of 0.18 Ca^{2+}/rho (6 experiments). In these experiments, the mean of the observed light-correlated Ca^{2+} change was a release of 3 ± 20% (S.D.), indicating no consistent light-activated Ca^{2+} release with light exposures that photoactivated either 0.1% or 8% of the visual pigment. Thus, no Ca^{2+} release was detected even in intact photoreceptors.

If light-activated Ca^{2+} release actually occurred in our experiments, the release must have been so small that it was masked by the variability in the light-correlated Ca^{2+} change observed between experiments. Given the extent of this variability, an upper bound can be estimated for the Ca^{2+} release. The standard deviation of the variability is about 30% of the disc content for all the experiments on isolated outer segments (Table II) and is 20% for the experiments in which the outer segments remained attached to the retina. Thus, flash exposures that photoactivated less than a few percent of the rhodopsin must have released less than 20—30% of the total Ca^{2+} content. This upper limit is equivalent to a release of less than 0.04 Ca^{2+}/rho ($\approx 10^5$ Ca^{2+} per disc). Since this limit set by experimental variabilities exceeds the release required by the Ca^{2+} hypothesis, the negative result of this investigation does not constitute a crucial test of the hypothesis. Given our observed Ca^{2+} content of each disc ($2 \cdot 10^5$) and the minimum release of Ca^{2+} required by the hypothesis (10^2—10^3), our experimental detection limit would have to have been lowered by more than two orders of magnitude to rule out the Ca^{2+} hypothesis (from ≈20% to ≈0.1%). In view of the variabilities we observed, it seems likely that an entirely

different experimental approach will be needed to achieve such a low detection limit.

Concurrent with our investigation, other studies of light-activated Ca^{2+} release have been reported. In the above article by Hendriks et al. [20], a release equivalent to about 5 Ca^{2+}/rho was reported for discs in which 50% or 85% of the rhodopsin was photoactivated either before or after a hypotonic shock. Liebman, on the other hand, observed a much smaller release, equivalent to about 0.07 Ca^{2+}/rho, after completely bleaching fragmented rod outer segments [9]. The detection limit in our experiments was sufficiently low to detect releases of similar magnitudes, had they occurred. Several investigators have studied Ca^{2+} release with radioactive tracers. Both Mason et al. [29] and Weller et al. [12] reported an increase in the rate of $^{45}Ca^{2+}$ efflux upon the illumination of bovine rod discs, which had been previously loaded with $^{45}Ca^{2+}$. However, such a light effect could not be demonstrated by Sorbi and Cavaggioni in comparable tracer experiments [30]. A key difference between our experiments and those of previous workers is that our experiments were designed to carefully investigate Ca^{2+} releases at low light exposures. We reduced the overall exposure of the outer segments to light as much as experimentally feasible so that only a fraction of the discs could have absorbed even one photon before the light stimulus was delivered. Just as importantly, we used light exposures that photoactivated even less than 1% of the rhodopsin. Although our strongest light exposures did not bleach more than a few percent of the pigment in the outer segments, such stimuli are more than adequate to elicit saturated peak responses in amphibian rods [31]. For testing the Ca^{2+} hypothesis, low light exposures are essential since the hypothesis requires the release of a large number of Ca^{2+} per photoactivated rhodopsin molecule [7,8, 24]. Such a stoichiometry can be observed only at low light levels. It is for this reason that the Ca^{2+} releases observed by others are equivocal results in terms of the hypothesis: Mason et al. [29] reported a release of 1 Ca^{2+}/photoactivated rhodopsin, and from the data of Hendriks et al. [20] and Liebman [9] we calculate a stoichiometry of 3 and 0.07, respectively. Since the Ca^{2+} content of discs is only 0.1 Ca^{2+}/rho, had we bleached 100% of the pigment in our experiments, we could have released at most 0.1 Ca^{2+}/photoactivated rhodopsin. On the other hand, exposures that photoactivated 0.1% of the pigment, or less, could have potentially released 10^2, or more, Ca^{2+}/photoactivated rhodopsin. The demonstration of the release of such a large number of Ca^{2+} per photoactivated rhodopsin molecule remains a crucial test of the Ca^{2+} hypothesis.

In conclusion, our results on the Ca^{2+} content of rod outer segments and discs are in reasonable agreement with the reports of Yoshikami and Hagins [19] and Liebman [9], and indicate that both rod outer segments and discs contain enough Ca^{2+} to satisfy the Ca^{2+} hypothesis. However, we feel that given our results and other published findings, the light-activated release of a large number of Ca^{2+} per photoactivated rhodopsin molecule as required by the hypothesis, has yet to be established.

Acknowledgements

This investigation was supported in part by a National Institutes of Health Training Grant to EZS and by a National Institutes of Health Research Grant from the National Eye Institute to RAC.

References

1 Baylor, D.A. and Fuortes, M.G.F. (1970) J. Physiol. 207, 77—92
2 Yoshikami, S. and Hagins, W.A. (1971) Biophys. J. Abstr. 11, 47a
3 Hagins, W.A. (1972) Annu. Rev. Biophys. Bioeng. 1, 131—158
4 Szuts, E.Z. (1975) Ph.D. Thesis, The Johns Hopkins University
5 Shichi, H., Lewis, M.S., Irreverre, F. and Stone, A.L. (1969) J. Biol. Chem. 244, 529—536
6 Yoshikami, S., Robinson, W.E. and Hagins, W.A. (1974) Science 185, 1176—1179
7 Hagins, W.A. and Yoshikami, S. (1974) Exp. Eye Res. 18, 299—305
8 Wormington, C. and Cone, R.A. (1975) Biophys. J. Abstr. 15, 171a
9 Liebman, P.A. (1974) Invest. Ophthal. 13, 700—701
10 Michaylova, V. and Ilkova, P. (1971) Anal. Chim. Acta 53, 194—198
11 Hemminki, K. (1975) Vision Res. 15, 69—72
12 Weller, M., Virmaux, N. and Mandel, P. (1975) Nature 256, 68—70
13 Liebman, P.A. (1972) Biophys. J. Abstr. 12, 99a
14 Harosi, F.I. (1975) J. Gen. Physiol. 66, 357—382
15 Robinson, W.E., Gordon-Walker, A. and Bownds, D. (1972) Nat. New Biol. 235, 112—114
16 Thiers, R.E. and Vallee, B.L. (1957) J. Biol. Chem. 226, 911—920
17 Scarpa, A., Baldassare, J. and Inesi, G. (1972) J. Gen. Physiol. 60, 735—749
18 Hagins, W.A. and Yoshikami, S. (1975) Ann. NY Acad. Sci. 234, 314—325
19 Yoshikami, S. and Hagins, W.A. (1976) Biophys. J. Abstr. 16, 35a
20 Hendriks, T., Daemen, F.J.M. and Bonting, S.L. (1974) Biochim. Biophys. Acta 345, 468—473
21 Chabre, M. and Cavaggioni, A. (1975) Biochim. Biophys. Acta 382, 336—343
22 Korenbrot, J.I., Brown, D.T. and Cone, R.A. (1973) J. Cell Biol. 56, 389—398
23 Falk, G. and Fatt, P. (1973) J. Cell Sci. 13, 787—797
24 Cone, R.A. (1973) in Biochemistry and Physiology of Visual Pigments (Langer, H., ed.), pp. 275 282, Springer-Verlag, Berlin Heidelberg
25 Szuts, E.Z. and Cone, R.A. (1974) Fed. Proc. Abstr. 33, 1471
26 Korenbrot, J.I. and Cone, R.A. (1972) J. Gen. Physiol. 60, 20—45
27 Bownds, D. and Brodie, A.E. (1975) J. Gen. Physiol. 66, 407—425
28 Cobbs, W.H. and Hagins, W.A. (1974) Fed. Proc. Abstr. 33, 1576
29 Mason, W.T., Fager, R.S. and Abrahamson, E.W. (1974) Nature 247, 562—563
30 Sorbi, R.T. and Cavaggioni, A. (1975) Biochim. Biophys. Acta 394, 577—585
31 Fain, G.L. (1976) J. Physiol. 261, 71—102