Light-dependent Ca\(^{++}\) content of rod outer segment disc membranes. P. A. LIEBERMAN.

Retinal rod outer segment disc membranes are found to bind Ca\(^{++}\). The Ca\(^{++}\) bound increases with that in the medium and after light exposure. The use of EGTA in the medium to prevent re-entry of released Ca\(^{++}\) unmasks a light-induced Ca\(^{++}\) loss from the disc membranes. Released Ca\(^{++}\) may be accompanied by phosphate. Our results are consistent with the Ca\(^{++}\) coupling hypothesis of visual transduction.

Calcium ions have been implicated as the coupling agent in a variety of biologic transductions. In vertebrate visual receptor cells, several experiments strongly imply that calcium may be released from a storage site such as the rod disc membranes (lamellar sacs) by the action of light. The released calcium is presumed to diffuse from the sacs to the rod envelope membrane where it is able to reduce sodium permeability and cause the increased membrane polarization which culminates outer segment transduction.

The purpose of the present experiments was to determine whether calcium is present in rod outer segments in significant quantity, whether this quantity is affected by light exposure and, ultimately, whether the kinetics of storage and release (if light sensitive) might be directly related to the time course of excitation and dark adaptation of rods.

Eyes were freshly obtained from frogs dark-adapted overnight. Cattle eyes were collected at a local abattoire from animals killed in dim light. These were placed in a light-tight box at room temperature for one hour before being transferred to ice. Retinas were removed in dim red light and collected in pH 7 phosphate-buffered ice cold 40 per cent sucrose. These were diluted with Ringer's solution to 23 per cent sucrose after one hour of soaking and centrifuged at 30,000 g for 15 minutes at 0° C. The retinal pellets were dispersed in fresh 40 per cent sucrose medium by vortexing and outer segments further dislodged and fragmented by syringing through a 25-gauge needle. Ringer's solution was layered over the sucrose suspension and the material centrifuged as before to yield a rod outer segment (ROS) fraction floating at the sucrose-Ringer interface (standard purification technique). After a second flotation, collected ROS were washed by resuspending and then pelleting at 30,000 g (15 minutes) from media consisting, respectively, of pH 7.2 phosphate-buffered Ringer (0.5 mM Ca\(^{++}\)), Ca\(^{++}\)-free Tris Ringer (2 mM Ca\(^{++}\) by assay) and Ca\(^{++}\)-free EGTA-Tris Ringer (1 mM EGTA). Neither of the "Ca\(^{++}\)-free" Ringer's contained phosphate. All Ringer solutions contained glucose (3.3 mM) and Mg\(^{++}\) (3 mM). Ordinary phosphate-buffered Ringer's was used in the preparative flotation steps.

The surface of the final pellet and centrifuge tube was rinsed with a few drops of deionized water. The pellet was then suspended in 1 ml. of ion-free water, transferred to a weighed silica crucible, dried at 100° C. for 1 to 2 hours, and re-weighed to a constant weight. The weight difference was recorded as sample dry weight. The dried pellet was then ashed in a crucible at 575° C. for 16 hours prior to calcium determination on a Baird-Atomic atomic absorption spectrometer. Accurately determined volumes of 0.1 N HCl/0.01 N SrCl\(_2\) were used with serial dilution until readings were on scale and behaved linearly upon subsequent dilution. For samples labeled "dark," all procedures were carried out in darkness or in dim red light until the final pellet was obtained. Samples labeled "light" were illuminated with white light from a 60-watt tungsten desk lamp placed one foot away for five minutes. The sample was suspended in the designated experimental Ringer's solution during this treatment. The initial pink color of suspensions vanished completely during light exposure. Calcium content of samples was calculated as nanomoles calcium per milligram of dry weight and per milligram of protein on the basis of previously known composition data for ROS. Aliquots of purified ROS examined by acrylamide gel electrophoresis and scanned at 280 nm showed 80 ± 10 per cent of integrated absorption to occur at 36,000 to 40,000 daltons, known previously to be rhodopsin.

Results are summarized in Table I. Several features of ROS Ca\(^{++}\) content are immediately evident. Calcium content of ROS is dependent upon calcium concentration of the experimental medium. In 0.5 mM Ca\(^{++}\) medium, ROS Ca\(^{++}\) content (~ 40 nmol per milligram of protein) is within a factor two to five of that found in muscle sarcoplasmic reticulum. ROS protein is 80 per cent rhodopsin. The molecular weight of rhodopsin is about 40,000 daltons. These figures yield the result that about two Ca\(^{++}\) per rhodopsin are associated with frog ROS lamellar sacs when isolated from 0.5 mM Ca\(^{++}\) medium and about one Ca\(^{++}\) per rhodopsin, in 2 mM Ca\(^{++}\) medium.

ROS lamellar sacs prepared as described herein have been shown to retain their characteristic flattened morphology. The intradisc space has been shown to be only 5 to 15 Å thick. The rhodopsin molecules imbedded in the disc membranes are known to have an average nearest neighbor distance of 70 Å. These values imply that intradisc Ca\(^{++}\) is about 0.1 M if confined to a 10 Å space. Both the Ca\(^{++}\) content measured and the calculated Ca\(^{++}\) concentration are clearly only lower limits since many of the sacs in our prepara-
Table I. Calcium content of ROS disc membranes (nanomoles per milligram of protein)

<table>
<thead>
<tr>
<th>Medium</th>
<th>Frog</th>
<th>Cattle</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Dark</td>
<td>Light</td>
</tr>
<tr>
<td>0.5 mM Ca++</td>
<td>38*</td>
<td></td>
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<tr>
<td>2 µM Ca++</td>
<td>15 ± 1</td>
<td>26 ± 1</td>
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<tr>
<td>1 mM EGTA (&lt; 10⁻⁸ M Ca++)</td>
<td>4 ± 0.3</td>
<td>2.6 ± 0.4</td>
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*Single measurement.

The studies presented above are sufficiently consistent with the Ca++ coupling hypothesis of visual transduction. The high concentration implied by these considerations suggests that ROS Ca++ must be membrane bound just as in sarcoplasmic reticulum. It is of interest that in the absence of EGTA, ROS Ca++ is always higher in the illuminated than in the dark-adapted preparations. Although at first surprising, this result is not inconsistent with the Ca++ coupling hypothesis. As in muscle sarcoplasmic reticulum, once Ca++ release has been triggered, a Ca++ uptake mechanism is necessary to restore dark adaptation in ROS or relaxation in muscle. Because of the long time delay between light exposure, and separation of the ROS membranes from the medium, it was not possible to demonstrate light-induced Ca++ release in the presence of this uptake mechanism even in the 2 µM Ca++ medium. One millimolar of EGTA was, therefore, added both to complex the 2 µM Ca++ in the medium and to trap any additional Ca++ released to the medium upon illumination. (EGTA is a quadrivalent anion assumed not to penetrate membranes.) EGTA appears to have reduced both dark and light Ca++ content, suggesting Ca++ leakage or exchange from the ROS membrane vesicles. More importantly, illumination is now seen to cause a measurable loss of Ca++ from the membranes. The light-dark Ca++ difference for release in EGTA is only 0.2 Ca++/rhodopsin but the true value may be at least 4- to 5-fold higher (e.g., 1 Ca++/rhodopsin), i.e., the factor by which the Ca++ content of the pellet in 2 µM Ca++ medium exceeds that in EGTA medium.

It is interesting to note that Ca++ determinations on supernates in these experiments did not behave linearly until four-fold dilution in SrCl₂. Calcium in the form of microcrystalline calcium phosphate is known to produce such an effect. Since our media contained no phosphate, however, such a complex could only have come from the rod disc themselves.

The preliminary results presented above are sufficiently consistent with the Ca++ coupling hypothesis of visual transduction to merit more thorough investigation of a Ca++ pumping mechanism (ATP dependence, ATPase activity, and ion specificity), proportionality of Ca++ release with fraction of visual pigment bleached, Ca++ leakage rate, and dependence of release mechanism on other ions. Such studies are now in progress.

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REFERENCES

Composition of asteroid bodies. WAYNE MARCH, DAVID SHOCH, AND RICHARD O'GRADY.

The human asteroid body by scanning electron microscopy is surrounded by a tightly adhering network of collagen fibrils. Smaller "satellite" asteroid bodies appear on and partially submerged in the surface of the larger body. Transmission electron microscopy reveals electron-opaque particles symmetrically arranged in these satellite asteroids. X-ray spectroscopy demonstrates the presence of calcium, sulfur, and phosphorus.