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Mg^{2+}-ATP induces filament growth from retinal rod outer segments with disrupted plasma membranes

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Mg^{2+}-ATP produces a large decrease in near-IR light scattering when added to suspensions of rod outer segments (ROS) when the plasma membranes have been disrupted by a gentle dialysis procedure. When this process is studied by light microscopy with video-enhanced image contrast, the Mg^{2+}-ATP-dependent signal is seen to be associated with the formation of filaments which extend only from those ROS lacking plasma membranes. Both the IR light scattering signal and filament growth are inhibited by vanadate and DCCD but not by colchicine, colcemid or cytochalasins.

Mg^{2+}-ATP; Cytoskeleton; Filament growth; Rod outer segment; Light scattering; Vision

1. INTRODUCTION

The mechanism of visual transduction is an archetype for many hormone receptors [1] and some neurotransmitter responses [2]. Understanding processes which occur in such complex membrane-protein systems requires a concerted application of many biophysical and biochemical tools. Light scattering is one such tool which has been valuable in studying the enzyme cascade of visual transduction [3–5].

Uhl et al. [6] first showed that an Mg^{2+}-ATPase caused a large IR light scattering change in suspensions of bovine ROS. This occurred in the dark (without stimulation of the visual pigment) and was called the A_D signal. Thacher [7] observed a similar signal in toad rods and presented evidence that the signal was not due to disk swelling. However, the mechanism responsible for the A_D signal has not been established.

Lewis et al. [8] reported a large-amplitude IR light scattering signal stimulated by visible light in the presence of GTP and proposed that the signal was due to changes in disk membrane aggregation. Caretta and Stein [9], using light and electron microscopy, showed that this large-amplitude GTP- and light-dependent signal was indeed due to aggregation of disk membranes. This result suggested that light microscopy might be useful in identifying the mechanism of the large Mg^{2+}-ATP-dependent light scattering signal.

Adding Mg^{2+}-ATP to suspensions of dialysed ROS results in a decrease in near-IR light scattering (increase in transmitted light, fig.1), which has very similar properties to the A_D signal as described by Uhl et al. [6]. Furthermore, video-enhanced light microscopy shows that our A_D signal is associated with the formation of filamentous processes which extend from only those ROS with disrupted plasma membranes.

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Abbreviations: AMP-PNP, adenylyl imidodiphosphate; DCCD, *N,N'*-dicyclohexylcarbodiimide; IR, infrared; ROS, rod outer segments

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2. MATERIALS AND METHODS

Bovine ROS were prepared by a modified version of a technique described by Uhl et al. [10]. Dark-adapted retinas (25–30) were collected within 2 h of slaughter and divided between two 30 ml screw-cap centrifuge tubes, each containing 6 ml of preparation buffer (600 mM sucrose, 14 mM glucose, 10 mM Na_2HPO_4, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 Kallikrein unit/ml Trasylol, and 1 mM dithiothreitol (DTT) added fresh, pH 7.3). The contents of each tube were vigorously sheared with a vortex-mixer for 2 min and forced through a 100 μm nylon mesh using trituration with the blunt end of a test tube. Retinal debris retained by the mesh was further washed with 6 ml preparation buffer. The ROS suspension which passed through the mesh was collected in a 26 ml cellulose nitrate centrifuge tube where it was underlaid with 15 ml of 31.4% sucrose in preparation buffer. The two tubes with gradients in them were centrifuged in a Beckman SW-28 rotor for 20 min at 20000 rpm. The carpet of ROS above the 31.4% sucrose solution was then collected with a syringe, carefully avoiding collection of the lower layer. The harvested carpets were pelleted by centrifugation for 15 min at 5000 rpm and finally resuspended in 2.5 ml of sodium light scattering buffer (NaLSB) (120 mM NaCl, 10 mM Tris, 1 mM $MgCl_2$, 1 mM DTT added fresh, pH 7.3). All operations were carried out using plastic labware under dim red light. The yield was typically 12 nmol rhodopsin per retina. *N,N'*-Didansylcystine staining showed that approx. 50–80% of the ROS had sealed plasma membranes [11].

ROS were dialysed in 1/4 inch tubing (Spectropore, M_r 12000–14000 exclusion) against two changes of dialysis buffer (1 mM EDTA, 1 Kallikrein unit/ml Trasylol, 1 mM DTT, pH 7.3) at a 1000:1 volume ratio. This disrupts the plasma membrane while retaining soluble proteins in the dialysis tubing. Ionic strength was restored by dialysis against NaLSB. Greater than 99% of the ROS prepared in this way had disrupted plasma membranes.

Light scattering changes were observed at 730 nm in a turbidity apparatus similar to that in [8]. Modifications included use of a photoflash instead of a laser for bleaching and a modified sample holder which allowed additions of reagents while turbidity measurements were in progress. Typical samples of dialysed material (4 mg/ml rhodopsin) displayed an apparent absorbance of 1.2 in a 2 mm path length sample cell.

All samples for microscopy were prepared under dim red light and studied using phase-contrast optics on a Leitz Dialux microscope with either an 800 nm narrow band-pass filter or a 680 nm cutoff filter. Samples were visualized by a Dage-MTI 65 MK II video camera equipped with a 1 inch RCA Ultricon II video tube and an Ikegami PM-175A video monitor. Recordings were made on a Hitachi VT-89A VHS video tape recorder for later study.

3. RESULTS

Fig.1 shows the change in near-IR light scattering that occurs when Mg^{2+}-ATP is added to a suspension of bovine ROS whose plasma membranes have been broken by gentle dialysis. Half-saturation of the signal occurs at about 400 μM ATP at a rhodopsin concentration of 100 μM. When viewed under phase-contrast microscopy using a video camera and a high-resolution video monitor, an array of filamentous projections is seen to extend from those ROS with ruptured plasma membranes (fig.2b). Visualization of these

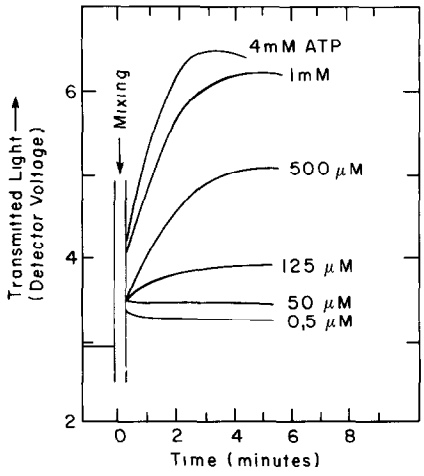

Fig.1. Effect of addition of different amounts of Mg^{2+}-ATP on the IR light scattering signal of dialysed ROS. Mg^{2+}-ATP was added to the final concentration indicated. The shaded region represents the variable data points that result during mixing of the samples. All samples were 100 μM rhodopsin.

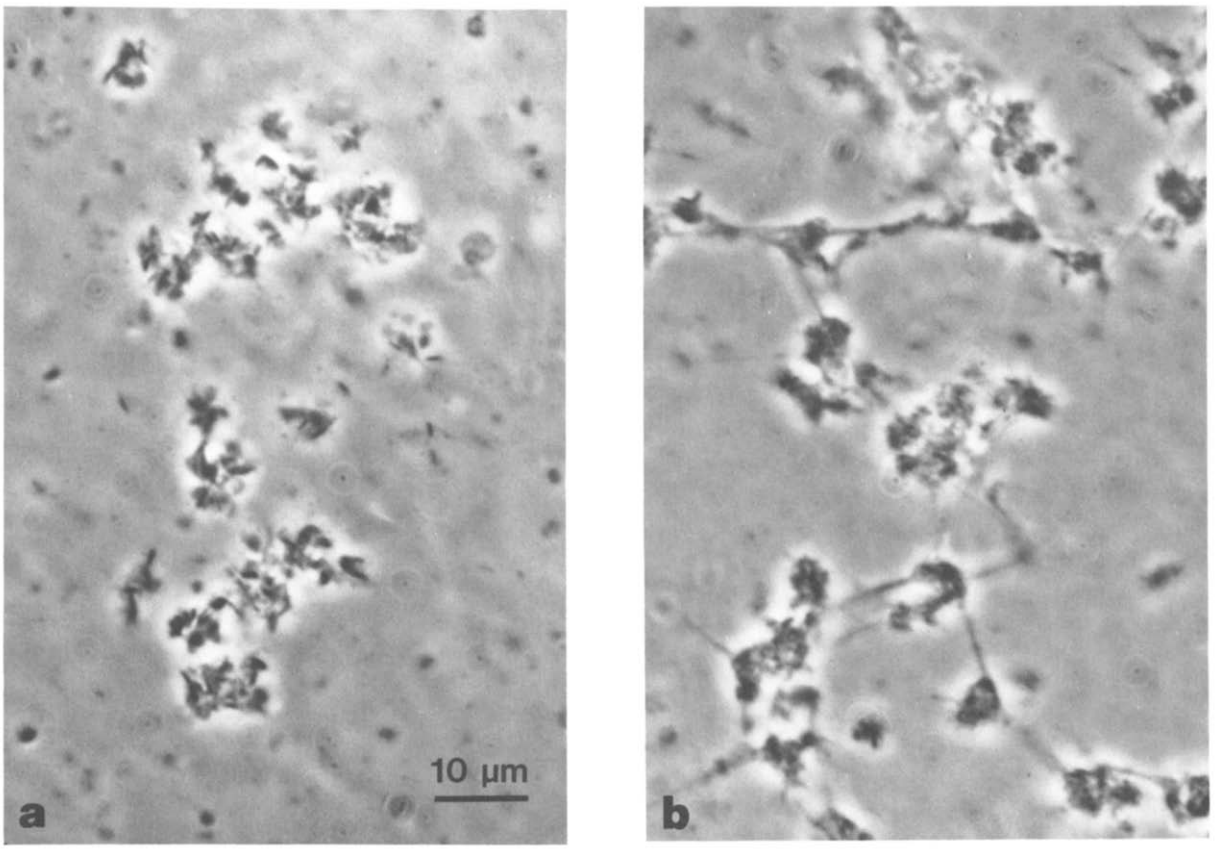

Fig.2. Phase-contrast micrographs of dialyzed ROS. (a) No added nucleotides; (b) 5 mM Mg^{2+}-ATP added to sample. Both samples were exposed to visible light to entangle filaments so they could be clearly photographed. Magnification, 1200 ×.

filaments requires the use of video technology because of the enhanced contrast that can be achieved [12]. In rare cases where the ROS plasma membrane has remained intact (< 1%) the ROS appears as a swollen sphere and no filaments are seen.

For microscopic observation, dilution of the initial suspension to 20 μM rhodopsin (1 : 5 dilution) is optimum. Fig.3 shows that this dilution attenuates and slows the light scattering signal. The initial concentration of material (100 μM rhodopsin) is too dense to observe structure between the broken ROS under the microscope. At 20 μM rhodopsin, the filaments are seen to grow from a time point at approx. 1.5 min after the addition of ATP (the time required to prepare the microscope slide and adjust for viewing), and reach a maximum length and density at 4–5 min. This time matches the time it takes the light scattering signal to reach maximum amplitude (fig.3). At low ATP concentration (< 1 mM) the filaments are very thin (sub-micrometer diameter) and form an extensive array around the disrupted ROS. Although relatively easy to visualize on an optimally adjusted video monitor, these thin filaments are very difficult to capture by still photography because of their rapid movement and low contrast. Numerous optical methods were tried to generate still photographs including dark-field illumination, differential interference contrast optics and millisecond Xe flash illumination but these methods did not improve photographic clarity. At high Mg^{2+}-ATP concentrations (> 1 mM) the filaments appear to become thicker with time which may correspond to the slight drop in light scattering signal seen at 4 mM ATP (fig.1). If these ROS are then

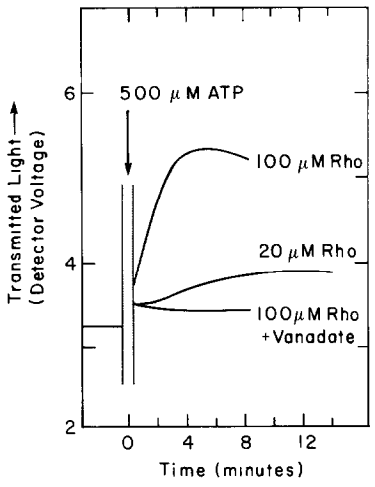

Fig.3. Additions of 500 μM Mg^{2+}-ATP to samples of dialysed ROS at indicated rhodopsin concentrations. Bottom trace is the result of addition of 500 μM Mg^{2+}-ATP after a brief incubation in 10 μM vanadate. If sample is incubated with 300 μM DCCD instead of vanadate, an identical result is seen.

Table 1

Comparison of IR light scattering signal and occurrence of filaments observed by light microscopy

Condition[a]	Light scattering signal[b]	Microscopy[c]
400 μM AMP-PNP	–	–
100 μM GTP	–	–
500 μM ATP	+	+
500 μM ATP after		
300 μM DCCD	–	–
10 μM vanadate	–	–
600 μM colchicine	+	+
600 μM colcemid	+	+
0.3 mg/ml cytochalasin B and D	+	+

[a] Final concentration of indicated reagent added to the dialysed sample
[b] Presence (+) or absence (–) of light scattering signal
[c] Presence (+) or absence (–) of filaments observed by video microscopy

irradiated with visible light, the filaments associate and tangle and are much easier to photograph (fig.2b).

Bleaching of a sample at the peak of our A_D signal results in a small light-induced increase in light scattering which has the character of the G^+ signal [8]. Presumably GTP to support a G^+ signal is produced by nucleotide diphosphokinase from ATP and the residual GDP which is present (~6 μM GDP found by HPLC, not shown). Full amplitude of the G^+ signal requires added GDP with dark incubation or $\geq$10 μM GTP (unpublished). The amplitude of the G^+ signal is much smaller in dialysed preparations compared to sonicated material, and represents a small fraction of our A_D amplitude.

As shown in fig.3 and summarized in table 1, vanadate (10 μM), an inhibitor of ATPases with phosphorylated intermediates [13], blocks both the light scattering signal and the formation of filaments in the presence of 500 μM ATP. DCCD (300 μM) also blocks both phenomena. There is neither a light scattering signal nor filament growth when the non-hydrolyzable ATP analogue AMP-PNP is added to the ROS suspension.

The light scattering signal was greatly attenuated but not eliminated by 0.1% Triton X-100, a concentration that would be expected to disrupt any transmembrane ion gradients. As indicated in table 1, a variety of cytoskeletal inhibitors of actin and tubulin polymerization were shown to have no detectable influence on the light scattering signal or filament formation. When GTP was added, in the absence of ATP and visible light, there was no IR light scattering signal and no filament formation observed.

4. DISCUSSION

The use of a video camera and monitor in combination with the light microscope has allowed us to visualize filaments which form in our samples upon the addition of Mg^{2+}-ATP. Without the contrast enhancement provided by this video technique these subcellular components are nearly impossible to see.

The dialysis procedure used here gently breaks the plasma membrane in greater than 99% of the ROS, improving the optical properties of the samples for light scattering [14]. This dialysis technique also provides for uniform access to the interior of the ROS, retains critical proteins in-

volved in phototransduction, and depletes the endogenous nucleotides. GDP is the only nucleotide present in significant amounts in these dialysed preparations as measured by HPLC (unpublished). Our A_D signal was consistently obtained in these preparations. Where sonication rather than dialysis was used to disrupt the plasma membrane of the ROS, the A_D signal was obtained only on occasion. Sonication of dialysed material does not eliminate the A_D signal, indicating that sonication alone does not damage the response. Thus, the depletion of endogenous nucleotides by dialysis appears to be the critical feature of our preparation that allows us to obtain consistent A_D signals.

The parallel between the observance of the light scattering signal and the presence of the filaments under specified conditions, as presented in table 1, leads us to believe that the filaments are directly associated with the changes seen in the light scattering signal. When samples of material were taken directly out of the light scattering cuvette during the A_D signal measurement and placed under the microscope, filaments could be seen. No filaments were seen in control samples without added ATP (fig.2). Filament growth and the decrease in light scattering are not stimulated by either AMP-PNP or GTP, while vanadate inhibits both processes. Thus, it appears that these processes require ATP hydrolysis. The Mg^{2+}-ATP-induced signal reported here has a half-saturation at about 400 μM Mg^{2+}-ATP compared to 10 μM for the A_D signal of Uhl et al. [6]. We attribute this difference to the much higher membrane concentration of our preparations (100 μM vs 1 μM rhodopsin used by Uhl et al.), a contention supported by other work where Uhl [15] found that the Mg^{2+}-ATP required for half-saturation increased linearly with membrane concentration. Other features of the signal are identical. Evidently a comparable amount of ATP per ROS is required to reach half-saturation in the two systems.

Usukura and Yamada [16] have shown a filamentous mesh-like network between ROS in the extracellular matrix. It is not likely that this material contributes to our signals since sealed ROS have no ATP-induced filament growth. Other electron microscopic studies by Usukura and Yamada [16] and Roof and Heuser [17] show the presence of filament-like connections between adjacent disks and between the disks and the plasma membrane. There is also a mesh-like network parallel to and just inside the rod plasma membrane. Numerous types of cytoskeletal components have been localized in ROS, including actin, fodrin, tubulin and 'microtubule-like' filaments [18–21]. However, inhibitors of actin and tubulin polymerization have no effect on our ATP-induced signal. Bert and co-workers [22,23] report that cytoskeletal inhibitors modify aspects of the electrophysiological response of ROS to light. Perhaps the filaments we observe reflect a dynamic interaction between cytoskeletal components and the enzyme cascade of visual transduction. This report, in combination with the data of Bert et al., suggests that cytoskeletal elements in ROS should be given far more detailed study to assess their importance in retinal physiology.

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