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ATP Causes a Structural Change in Retinal Rod Outer Segments: Disc Swelling Is Not Involved

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Summary. Mg^{2+}-ATP was found to produce a 15 to 30% drop in the turbidity of suspensions of broken retinal rod outer segments from the toad *Bufo marinus*, prepared by washing or flotation in sucrose. This *in vitro* process has a half-time of about two minutes and appears to be irreversible. It is not affected by the bleaching of rhodopsin. Direct measurements show that the drop in turbidity is not due to swelling of the disc internal space measured in outer segments recovered by centrifugation. Instead, the total packed volume of the outer segments increases following incubation in Mg^{2+}-ATP. Under the specific conditions of these experiments, the total pellet volume increase was $26 \pm 22\%$ (13 experiments) when corrected for the percent of rhodopsin recovered in the centrifugal pellet. The magnitude of the ATP effect on turbidity suggests that the majority of the discs are involved in some kind of structural change. Vanadium in the +5 oxidation state (vanadate) is an inhibitor of the Mg^{2+}-ATP effect on turbidity at a half-maximal concentration of 0.2 to 0.4 μM, and inhibition is rapidly reversed by norepinephrine, which complexes vanadate. A Mg^{2+}-ATPase activity in extensively washed outer segment membranes, previously shown to be activated as much as twofold following light exposure of the membranes, is not sensitive to vanadate at the concentrations which block the ATP-dependent change of turbidity.

Key Words retinal rod outer segment · ATP · vanadate inhibition · structural change · disc internal space · light-scattering

Introduction

The retinal rod outer segment is filled with a highly ordered array of flattened discs (Daemen, 1973) which are physically linked to each other at their rims, and remain in stacks when the outer segment is fragmented (Cohen, 1970; McConnell, 1975; Roof & Heuser, 1982). The disc membrane contains primarily the visual pigment rhodopsin and encloses an osmotically active space, virtually impermeable to all ions, except those which cross the membrane in a lipid-soluble form (Brierley et al., 1968; Uhl et al., 1980). Suspensions of toad outer segment fragments exposed to Mg^{2+}-ATP showed a very substantial drop in turbidity or light-scattering, a phenomenon which has also been described in bovine ROS (Uhl, Borys & Abrahamson, 1979*a*, *b*). This novel effect was much greater than expected for swelling of the internal space due to energy-dependent transport of ions or molecules to the disc interior. It is demonstrated here by direct physical means that no significant change of disc internal volume takes place during Mg^{2+}-ATP incubation. The light-scattering or turbidity change must therefore be explained in other ways, including structural alterations of the outer segment disc stack as a whole. Other biochemical aspects of this novel effect of ATP on outer segments are also described. These results have previously appeared in summary form (Thacher, 1980).

Materials and Methods

The sources of most reagents have been described earlier (Thacher, 1978). ^3H_2O (Amersham, Arlington Heights, Ill.) was a gift of B. Saver (Harvard, Department of Chemistry); $^{86}Rb^+$, Aquasol, and Protosol (New England Nuclear, Boston, Mass.), ^{14}C-sorbitol (ICN, Irvine, Calif.), hexokinase Type C-302 (Sigma, St. Louis, Mo.), and ammonium vanadate (Fischer, Fair Lawn, N.J.) were purchased. DNAase came from Mann Research Laboratories. Oligomycin (a mixture of the A, B and C forms) was a product of Sigma.

A23187 was a gift of R. Hamill, Eli Lilly; gramicidin (gramicidin A primarily, recrystallized) was a gift of E. Blout, Harvard Medical School; valinomycin was purchased from Sigma. A recrystallized preparation of dinitrophenol was available in this laboratory. Ionophore activity was assayed by outer segment swelling (Brierley et al., 1968).

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Abbreviations: Hepes, N-2-hydroxymethylpiperazine-N′-ethanesulfonic acid; EGTA, 2,2′-ethylenedioxybis(ethyliminodiacetic acid); EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PK, pyruvate kinase; PEP, phosphoenol pyruvate; AMP-PNP, adenylyl imidodiphosphate; OD, optical density; ROS, rod outer segment; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; PMSF, phenylmethylsulfonyl flouride.

Isolation of Outer Segments

Retinas were prepared from *Bufo marinus* (Thacher, 1978), and all preparations and assays were done under dim red light unless otherwise stated. After vigorously shaking two to six retinas in 1 to 2 ml of 38% sucrose (wt/wt) (buffered in 10 mM Na-Hepes, pH 7.3, 5 mM KCl, 50 μM EDTA, 0.2 mM $MgCl_2$, 60 μM $CaCl_2$, 1 mM DTT), the outer segments were forced through a 30-μm pore size nylon mesh in a Millipore Swinnex-25 filter holder to retain the retinal fragments, and the filter was washed with additional 38% sucrose. The suspension was overlaid with 24% sucrose in a Beckman SW-50 rotor tube and centrifuged 10 min at 10,000 rpm. Alternatively, the purification was by isopycnic sedimentation on a 24 to 38% sucrose gradient. Few intact outer segments are left, as is obvious by phase contrast microscopy.

The outer segments in sucrose were diluted two- to three-fold with the same buffer as was used in the the sucrose solutions, spun down at 12,000 rpm for 15 min, taken up gently in buffer A (122 mM NaCl, 10 mM Na-Hepes, pH 7.3, 20 mM KCl, 0.5 mM $MgCl_2$, 6 μM $CaCl_2$, 1 mM DTT), and dispersed with a snug but not tight-fitting Teflon homogenizer in a glass test tube. Vigorous dispersion of outer segments by a Pasteur pipette that was not flame-tempered diminished their total light-scattering and their response to ATP also. Outer segments were also prepared on a metrizamide isosmotic density gradient (Thacher, 1978) instead of sucrose and washed in buffer A. All solutions and preparations were kept under argon or nitrogen gas until use.

Light-Scattering or Turbidity

Light-scattering or turbidity, as optical density, was measured in a Zeiss PMQ-II spectrophotometer at a wavelength of 750 nm with a <620 nm cutoff filter in place. All assays were done at room temperature in approximately 0.4 ml with a concentration of 30 to 80 μg rhodopsin $(ml)^{-1}$. Reagents were added to a cuvette, then mixed by gentle agitation with a Pasteur pipette whose tip was flame-polished.

Apparent Disc Internal Volume

Apparent disc internal volume is defined here as the difference between the total volume of an ROS pellet, measured with ^3H_2O, and the external space which is accessible to ^{14}C-sorbitol or $^{86}Rb^+$. Sorbitol (80 μM) and 20 mM KCl were present in all assays to minimize the nonspecific binding of the tracers. Outer segments, 0.6 ml with 0.3 to 0.5 mg rhodopsin $(ml)^{-1}$, were incubated at room temperature (with or without ATP) in buffer A in which the NaCl concentration was varied to change the osmolarity. 50 μl of radioactive tracers, at about 0.3 to 1.0×10^6 cpm/ml, were added to the suspension on ice after the incubation with ATP was finished. For measurements of disc permeability to sorbitol, the tracers were added at the start of the incubation.

200 μl aliquots of the suspension were placed in three 0.4 ml capacity polyethylene tubes (Walter Sarstedt and Co., Princeton, N.J.) and spun 40 s in a Beckman Microfuge B. The supernatants of the three tubes were pooled and saved, and rhodopsin concentration determined by light absorption (Thacher, 1978). The tubes were wiped clean of excess liquid with cotton-tipped applicators. The tip of the tube was cut off, and the pellet in the tip was partially solubilized with 10% Triton X-100 (Rohm and Haas, Bristol, Pa.), using the tip of a glass Pasteur pipette which had been drawn out over a flame and broken at its narrowest point. The tube tip and the glass tip which was used for agitation of the pellet were placed in a 15 ml counting vial with 0.5 ml 2:1 absolute ethanol:Protosol. After shaking, 5 ml Aquasol and 0.15 ml 2 M acetic acid were added, in that order. The radioactivity per volume of the ^3H_2O and ^{14}C-sorbitol tracers in the supernatant was used to calculate the volume accessible to each tracer in the pellet. The internal and total volumes of the pellet were corrected for unsedimented material according to the rhodopsin content of the supernatant.

ATP Hydrolysis

Preparation of the substrate, [γ-^{32}P]-ATP, and the assay method have been described (Thacher, 1978).

Results

Turbidity Change due to the Addition of ATP

When ATP was added to a preparation of broken outer segments in the presence of Mg^{2+}, a large reduction in turbidity took place over several minutes (Fig. 1, curve *a*), as measured by the optical density of the suspension at 750 nm. The total reduction in optical density was generally about 15 to 30%. The percentage change was relatively independent of the measuring wavelength between 650 nm and 1.1 μm although, as expected, the total light-scattering decreases at longer wavelengths (Uhl et al., 1980). After ATP was removed by the hexokinase-catalyzed phosphorylation of glucose (curve *b*), the turbidity change stopped, and the turbidity did not change back after as much as one hour's time. According to the curves *c* and *d* of the Figure, ADP had no effect on turbidity unless it could be phosphorylated to ATP by pyruvate kinase (PK) and phosphoenol pyruvate (PEP). Because ATP was continuously regenerated in *c* and *d* of Fig. 1, the tapering-off of the turbidity change cannot be due to its exhaustion. Bleaching the rhodopsin in these samples did not significantly alter the effect of ATP.

Adenylyl imidodiphosphate (AMP-PNP), a nonhydrolyzable analog of ATP, interfered when added at a comparable concentration to that of ATP, either before ATP addition, or after, as shown in Fig. 1, curve *e*. The K_m of ATP for the initial rate of the turbidity change was estimated to be about 40 ± 20 μM.

Neither 1 mM Mg^{2+}-GTP nor 0.5 mM Mg^{2+}-guanylyl imidodiphosphate (GMP-PNP) inhibited the turbidity change in the presence of 20 μM ATP, and Mg^{2+}-GTP alone had little or no effect in the assay. The turbidity change in bovine outer segments has a very specific requirement for ATP, also. Very rapid effects of visible light on light-scattering in bovine and frog outer segments are clearly distinct from the phenomena described

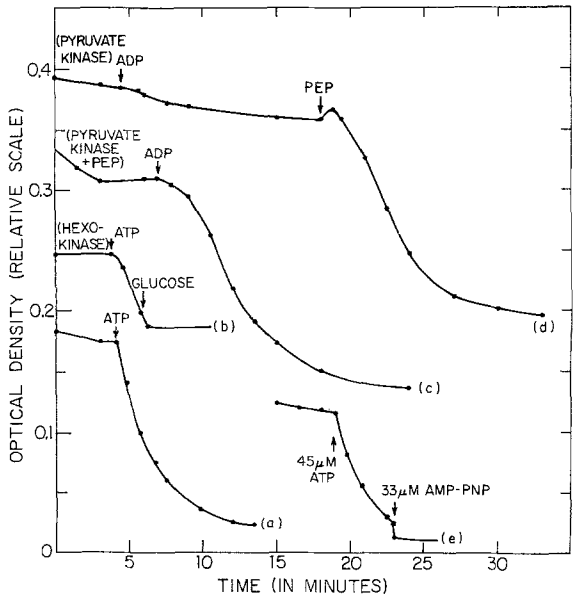

Fig. 1. Optical density (750 nm) of outer segment fragments at a rhodopsin concentration of 70 to 90 μg $(ml)^{-1}$, in 50 mM NaCl, 10 mM Na-Hepes, pH 7.3, 5 mM KCl, 1 mM DTT, 0.3 mM $MgCl_2$, and 0.1 mM EGTA. Initial sample OD's were 0.55 to 0.65. Precise additions for each trace were: (*a*) 0.27 mM Mg^{2+}-ATP; (*b*) hexokinase, then 0.27 mM Mg^{2+}-ATP, followed by 2 mM glucose; (*c*) pyruvate kinase and 0.75 mM PEP initially, then 0.3 mM Mg^{2+}-ADP; (*d*) pyruvate kinase initially, then 0.3 mM Mg^{2+}-ADP, followed by 0.75 mM PEP; (*e*) 45 μM Mg^{2+}-ATP, followed by 33 μM AMP-PNP. Each trace is replotted from a strip chart recorder. The interruptions during sample addition are filled in by interpolation

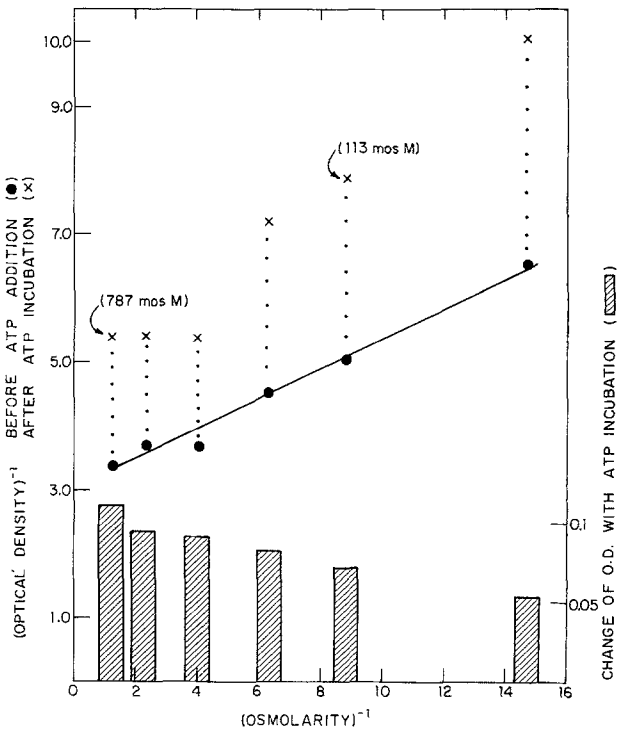

Fig. 2. Comparison of effect of osmolarity and ATP incubation on light-scattering from fragmented outer segments in suspension. Relation of light-scattering to osmotic strength: 40 μl samples in buffer A, with rhodopsin at 0.48 mg $(ml)^{-1}$, were diluted into 0.36 ml of 10 mM Hepes, pH 7.3, 5 mM $MgCl_2$, 0.1 mM EGTA, 5 mM KCl, 1 mM DTT, and variable NaCl, from 0 to 400 mM. The OD was recorded when samples initially stabilized after mixing in the absence of ATP (●). Each point is the average of two assays at a single osmolarity and the initial OD and osmolarity are plotted as their inverses. The actual osmolarity is noted at the top of the Figure in two cases. After addition of ATP (5 μl of 13 mM Mg^{2+}-ATP, 0.16 mM final concentration), the final OD was recorded several minutes later, after it had leveled off (×). A histogram of the absolute value of the decrease in OD is at the bottom of the Figure; the scale of the histogram is on the right

here, however (Uhl, Hoffman & Kreutz, 1978; Bignetti et al., 1980; Kuhn et al., 1981).

The ATP effect requires Mg^{2+}, but is not noticeably altered by varying the pH from 6.5 to 8.1. Ca^{2+} was somewhat inhibitory at 50 μM, but when reduced below 10^{-5} μM (using EGTA as a Ca^{2+} buffer) no change in the ATP effect was found. In some preparations, the monovalent cations Na^+ and K^+ were left out of all buffers, and the ATP response of the outer segment suspension was similar whether or not they were added back.

Outer segments prepared in sucrose consistently responded to ATP, although the magnitude of the decrease in optical density was variable, depending on the preparation. When purified on a metrizamide gradient, or by gentle shaking of the retina, no ATP response was observed. In two experiments, outer segments prepared on metrizamide were washed in sucrose and then became responsive to ATP.

Disc Volume Change

The reciprocal of the optical density or turbidity of broken outer segments at 750 nm is proportional to the reciprocal of the osmolarity of the medium (Brierley et al., 1968; McConnell, 1975; Uhl et al., 1980), as shown in Fig. 2. [Decreased turbidity as a consequence of the swelling of an osmotically active cell or isolated organelle is due to a decreased refractive index of the internal space (Sidel & Solomon, 1957; Tedeschi & Harris, 1958).] The magnitude of disc swelling necessary to explain the ATP effect can be predicted from the upper part of Fig. 2 (the lower part of the Figure is a histogram of the absolute value of the decrease in OD at each osmolarity). At 787 mOsm, for example, after ATP incubation, the final OD of a suspension of outer segment fragments is the same as expected for a similar suspension placed directly in a medium at 100 mOsm. Thus, in order to account for the effect of ATP entirely by disc

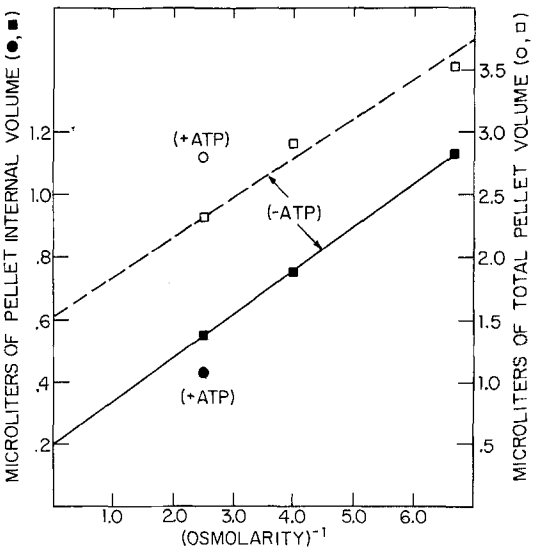

Fig. 3. Internal and total outer segment pellet volumes as a function of osmolarity and ATP. Assay as described in Materials and Methods: all samples were incubated for 6 min at 25 °C, and the final rhodopsin concentration after tracer additions was 0.35 mg $(ml)^{-1}$; assays were performed at 400, 250 and 150 mOsm; 1 mM Mg^{2+}-ATP was present during the incubation of one tube at 400 mOsm. The median internal and total volumes of the pellets of the three 200 µl aliquots used for each assay point were corrected for unsedimented material. Internal volumes (■, −ATP; ●, +ATP). Total pellet volumes (□, −ATP; ○, +ATP)

swelling, an eightfold increase of disc volume would have had to take place.

To test this possibility, the apparent disc internal volume was directly measured in a pellet of outer segment fragments. The data of Fig. 3 show that disc internal volume is sensitive to medium osmolarity but that the effect of ATP is small and probably not significant (*see* Table). As a control, outer segments from the preparation used in Fig. 3 were exposed to ATP in a cuvette at 400 mOsm. The decrease in turbidity was equivalent to the effect of an approximate threefold reduction in medium osmolarity. Therefore, although a change of disc volume can easily be induced under the proper conditions and measured, it does not accompany the light-scattering changes produced by ATP to a significant extent. The disc internal volume is much higher than expected from measurements on intact outer segments, as described in the appendix.

It did not appear that discs were damaged by ATP incubation, according to experiments using the internal volume assay. First, the slope of an internal volume *vs.* $(osm)^{-1}$ curve such as in Fig. 3 remained constant after ATP incubation. In this experiment, ATP was degraded to ADP (as in Fig. 1, curve *B*) after incubation at 400 mOsm. The outer segment fragments were then transferred to

Table. ATP effect on ROS disc internal volume and total volume

Ratio: $\frac{\text{ATP-treated}}{\text{control}}$	Mean	Standard error of mean	Significance[a]
Apparent disc internal volume	1.06	0.20	$0.2 < P < 0.4$
Total pellet volume per mass rhodopsin	1.26	0.22	$P < 0.005$
Rhodopsin recovery in pellet[b]	0.81	0.11	$P < 0.001$

[a] 13 experiments, 9 preparations of outer segments. Significance calculated according to the Student's *t*-test.
[b] In control pellets, an average of 77% total rhodopsin was recovered.

media of differing osmolarities for internal volume determinations. Second, outer segment discs did not significantly change their permeability to ^{14}C-sorbitol during ATP incubation at 25 °C. After a 15-min incubation, the apparent internal volume of the discs was reduced by 30% because of the ^{14}C-sorbitol leakage to the disc interior, and this leakage was the same in the presence of ATP.

Total Pellet Volume

The data of Fig. 3 show that the total volume of the centrifugal pellet recovered in the internal volume assay increases following ATP incubation. The values shown in Fig. 3 and used in the Table are corrected for the amount of rhodopsin recovered in the pellet, which was reduced an average of 81% in ATP-treated samples. Using corrected values, the total volume increase of 26% was a statistically significant one. If this correction for rhodopsin recovery in the pellet was not used, so that more experiments could be included in calculations of the average change of total volume, an 11% increase with a 21% standard error ($P < 0.005$ for 37 experiments on 26 preparations) was found.

Phase microscopy showed no obvious signs of aggregation or disintegration of stacks of outer segment discs in the presence of ATP. The size of the disc stacks in most preparations was one-tenth or less of the total mass of an outer segment. Break-up of the outer segment fragments to this point from larger fragments did not seem to alter the ATP effect. Either disc lysis in a very low osmotic strength buffer, or excessive agitation of the outer segment membrane suspension with a Pasteur pipette substantially reduced the ATP effect, however.

Inhibition by Vanadate

Biochemical analysis of the light-scattering effect continued with an effort to find an inhibitor. A number of compounds were tested without any effect: 1 mM PMSF, 100 μM DNDS, an inhibitor of anion transport in red blood cells (Barzilay & Cabantchik, 1979), 3′,5′-cyclic AMP or cyclic GMP at 0.25 mM each, 25 μg/ml of the following added together: colchicine, vinblastine and cytochalasin B, or DNAase I (30 μg/ml); oligomycin at 10 μg/ml had no effect on the toad membranes but is reported to cause partial inhibition of ATP-dependent light-scattering changes in bovine outer segments (Uhl et al., 1979*b*).

Ionophores added in the presence of their corresponding permeant ions (McLaughlin & Eisenberg, 1975) – valinomycin and dinitrophenol plus KCl, or gramicidin and A23187 plus NaCl, KCl, and $CaCl_2$ – had little or no effect on the ATP response. If permeant ions such as acetate were also included to cause swelling of the discs, an ATP response was still observed.

Vanadate, a very potent inhibitor of the (Na,K)-ATPase (Cantley et al., 1978*a*), was consistently inhibitory at micromolar levels. The onset of vanadate inhibition was rapid (Fig. 4), and the inhibition was also rapidly reversible by norepinephrine, which is known to complex to vanadate (Cantley et al., 1978*b*). Vanadate inhibition can take place in the presence or absence of an ATP-regenerating system. Free vanadate had an apparent K_I of 0.2 to 0.4 μM for inhibition of the light-scattering change (Fig. 5), and the apparent K_I was much higher in the presence of 0.5 mM norepinephrine. Two well-known ATPases which transduce energy into movement, myosin and dynein, are inhibited by vanadate. The inhibition of myosin requires ADP and is very slow to reverse when vanadate is removed (Goodno, 1979), so its involvement can be ruled out. Dynein ATPase and flagellar beating are superficially similar to the ATP effect (Thacher, 1980): the K_I for vanadate is 0.5 to 1.0 μM, the inhibition of both is easily reserved by norepinephrine (Gibbons et al., 1978), and furthermore, flagellar beating is activated with ATP but not GTP (Gibbons & Gibbons, 1972).

Outer segments, taken directly from a sucrose gradient but not washed, seemed less sensitive to vanadate, and inhibition was more variable. This may have been due to the inactivation of vanadate by reduction to the (+4) oxidation state, as occurs in other systems (Cantley & Aisen, 1979).

The effect of vanadate on ATPase activity in ROS is shown in Fig. 6 under the conditions of high NaCl similar to those of Fig. 5. Up to 5 μM vanadate had little effect on the increased ATPase activity which results from bleaching rhodopsin (Thacher, 1978). The ATPase activity sensitive to vanadate partly reflects the presence of the ouabain-resistant (Na,K)-ATPase of the toad

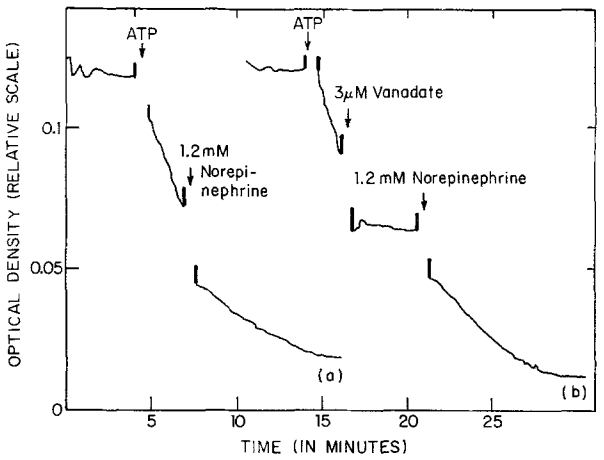

Fig. 4. Vanadate inhibits the light-scattering change. Samples were incubated as in Fig. 1, with these changes: 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM PEP and 4 U $(ml)^{-1}$ PK were present; reaction volume was 0.4 ml. Strip-chart recordings are retraced. Vertical marks show the opening and closing of the sample chamber to add reagents. (*a*) is the control, initial OD of 0.27 at 750 nm; (*b*) shows vanadate effect, initial OD 0.33. The turbidity change when adding reagents is not sample dilution, but disturbance of the membranes during mixing. Additions were: 1 μl 84 mM ATP, 10 μl 50 mM norepinephrine, and 1.2 μl 1 mM vanadate at the times shown

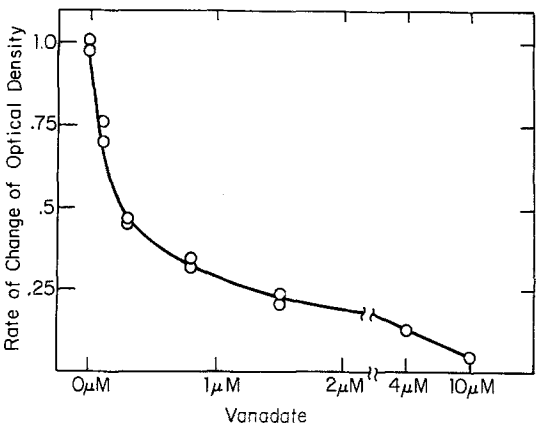

Fig. 5. Initial rate of ATP-dependent turbidity change as a function of vanadate concentration. The maximum initial slope, taken from curves such as those in Fig. 1, is shown as a fraction of the initial slope in the absence of vanadate. The reaction mixture containing the outer segment suspension was 0.38 ml of 10 mM Na-Hepes, pH 7.3, 5 mM $MgCl_2$, 100 mM NaCl, 5 mM KCl, 0.5 mM DTT, 0.1 mM EGTA, 0.4 mM PEP, 3 U $(ml)^{-1}$ PK. Vanadate (10 μl) was followed immediately by 10 μl 10 mM ATP to initiate the reaction. The final vandate concentrations are shown on the abscissa

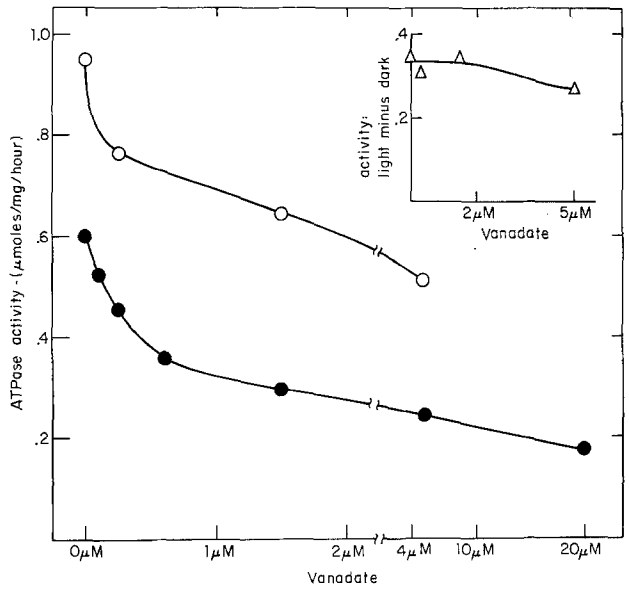

Fig. 6. Vanadate inhibition of ATPase activity. Outer segment membranes were washed in 1 mM Na-EDTA, 0.2 mM Tris-EDTA prior to assay. Each assay contained 0.12 mg $(ml)^{-1}$ rhodopsin, 0.25 mM ATP and 10 mM Hepes-Tris, pH 7.3, 5 mM $MgCl_2$, 50 mM NaCl, 5 mM KCl, 0.5 mM DTT, 0.1 mM EGTA, 0.3 mM PEP, 2 U $(ml)^{-1}$ PK, 10 μM ouabain, and efrapeptin. Vanadate was added to the membranes on ice, prior to assay. Time of assay: 15 min. Temperature: 29 °C. (●–●) unbleached ROS; (o–o) ROS bleached prior to assay; inset (Δ–Δ) difference in activity between bleached and unbleached membrane. Activity is measured in μmoles phosphate released (mg rhodopsin)$^{-1}$ $(hr)^{-1}$. Each point is the median of triplicates

(Flier et al., 1979) usually found in outer segment preparations (Thacher, 1981). ATPase activity sensitive to 1 μM vanadate was observed in other experiments, in the absence of sodium, but not analyzed in detail. Inhibition of a bovine ROS ATPase by 10 μM vanadate has also been reported (Uhl et al., 1982).

Conclusion

In terms of the area of its membranes, 99% of the amphibian rod outer segment is composed of discs (Daemen, 1973; Fain, 1976). Because the disc protein in light-scattering assays is at a very low concentration, the turbidity change at 750 nm which occurs during ATP incubation must be explained by a change in the morphology and therefore the light-scattering properties of the discs or clusters of discs rather than conformational changes in the outer segment proteins themselves. An obvious explanation for the drop in turbidity, the swelling of the discs in response to ATP, was ruled out by direct measurement of the disc internal volume, validating the suggestion of Uhl et al. (1979*b*) which was based on light-scattering studies of bovine outer segments. This conclusion is further supported by the fact that outer segment membranes remained responsive to ATP treatment even when they were permeable to many of the ionic species in the surrounding medium as a result of ionophore treatment.

Another kind of structural change, other than swelling of the disc internal space, must be responsible for the effect of ATP on the turbidity of the outer segment suspension. The increase in volume of the centrifuged outer segment pellet following ATP incubation, as well as the decreased sedimentability of the outer segment fragments, suggests that some alteration of disc morphology, or of the structures which hold the discs in stacks, underlies the decrease in turbidity of outer segment suspensions.

The ATP-effect might be explained in two ways. The less interesting would be that the nonspecific action of an ATP-dependent enzyme, possibly an impurity, degrades outer segment structure in some way. This possibility cannot be completely ruled out. Extensive breakage or dispersion of outer segment fragments is not seen by light microscopy, however, nor is protein degradation observed on SDS gels (*unpublished observations*). Also, discs retain their osmotic sensitivity and do not change their permeability to ^{14}C-sorbitol as a result of ATP treatment. These results suggest that the disc stack is not seriously damaged by ATP incubation.

The second possibility is that ATP action is related to an energy-dependent process of physiological importance to the outer segment. Because the changes in turbidity are 15 to 30%, and the outer segment fragments in the suspension are small in comparison to the size of the intact outer segment, specific structural alterations must take place throughout all disc stack fragments rather than in fragments derived only from the tip or base. The toad rod is 65 μm long (Fain, 1976), and the nine microtubule doublets of the rod cilium usually extend only about 2 μm into the outer segment (Rohlich, 1975; Young, 1976) so that their participation is unlikely. On the other hand, the direct links which have long been suggested to occur between disc rims (Falk & Fatt, 1969; Cohen, 1970; Papermaster et al., 1978), and have been recently visualized (Usukura & Yamada, 1981; Roof & Heuser, 1982), could logically mediate the effect of ATP on disc structure.

In vivo, the effect of ATP must be closely controlled given that 1 to 2 mM ATP and enzymes that regenerate ATP from ADP are present in the outer segment (Donstov, Zak & Ostrovskii, 1978; Robinson & Hagins, 1979). The sucrose wash must

either remove inhibitory substances or otherwise alter outer segment structure so that the ATP-dependent process can be observed, although in an unphysiological fashion. Several types of disc movement, which occur during outer segment turnover, have been described. One of them could possibly underlie the ATP effect. For example, compression and expansion in disc spacing take place during the light-dark cycle of disc addition to the outer segment (Besharse, Hollyfield & Rayborn, 1977). Two other processes related to outer segment turnover occur only at the rod base or tip: synthesis or formation of new discs from the plasma membrane (Steinberg, Fisher & Anderson, 1980), or pinching off of disc packets from the tip of the outer segment (Bok & Young, 1979). Machinery for one of them might be present throughout the outer segment in some form, to be manifested *in vitro* in an unregulated manner.

Two ATP-dependent reactions of outer segments can be ruled out as participating in the light-scattering change: the phosphorylation of rhodopsin, which is greatly activated by bleaching (Miller, Paulsen & Bownds, 1977), and the light-enhanced ATPase (Thacher, 1978), which is not inhibited by the vanadate concentrations of 1 μM or less which block the ATP-dependent turbidity change. An ATPase enzyme of toad ROS membranes, identified only by its phosphorylated intermediate, is sensitive to concentrations of vanadate as low as 1 μM (Thacher, 1981) and may be of importance. A rapid ATP hydrolysis on the time course of the light-scattering change that is inhibited by 10 μM vanadate has also been reported in bovine ROS (Uhl et al., 1982). Since outer segments of cattle (Uhl et al., 1979*a*, *b*) and *Rana pipiens* (*unpublished observations*) have responses to ATP like the toad's, which are inhibited by AMP-PNP and vandate, but not GTP, the ATP-dependent process described here is likely to be common to outer segments in all vertebrate species.

Appendix

The osmotically sensitive disc internal volume (Fig. 3) is described as follows:

V_m (apparent disc internal volume) $= V_o + K/$(osmolarity),

where K is the slope of the lower line in Fig. 3, and V_o is the y-intercept, or the internal volume extrapolated to infinite osmotic pressure. The osmotically sensitive internal volume at a certain osmolarity is derived from V_m by subtraction of V_o. At the osmolarity of toad Ringer's, about 250 mOsm (Fain, 1976), it is unexpectedly high: 6.8 ± 1.9 μl (mg rhodopsin)$^{-1}$ in five preparations, or 10- to 20-fold greater that what is expected from X-ray or neutron diffraction measurements of intact outer segments. Based on an upper estimate of 20 Å for the osmotically sensitive intradisc space (Chabre & Cavaggioni, 1975; Yeager, 1975), a rhodopsin concentration of 3.5 mM in the outer segment (Harosi, 1975), and disc spacing of 300 Å, the expected internal volume is 0.4 μl (mg rhodopsin)$^{-1}$. This magnitude of swelling in isolated frog discs has also been noted as a reduction in their density, as observed by isopycnic centrifugation (Szuts, 1980).

V_o is larger than expected also. In the five experiments it is 3.1 ± 2 μl (mg rhodopsin)$^{-1}$. The expected value of V_o should be the sum of two parts: one, an irreducible volume inside the disc, about 20 Å if the total spacing between lipid bilayers is 40 Å (Yeager, 1975; Saibil, Chabre & Worcester, 1976), and two, hydrogen exchange, about one proton per amino acid residue (Downer & Englander, 1977). The total of the two parts is 0.5 μl (mg rhodopsin)$^{-1}$. The high estimate of V_o could be an artefact due to some disc rupture at the lower osmolarities where V_m is determined, thereby reducing K and increasing V_o.

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