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MOLECULAR MECHANISMS OF PHOTORECEPTION

IV. Ca^{2+}-INHIBITED GTPase OF ROD OUTER SEGMENTS OF THE FROG RETINA

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Summary

Ca^{2+}-dependent GTPase activity is found to be present in the rod outer segments of frog retina. GTPase localization in rod outer segments is shown by fractionating the rod outer segment preparation in the sucrose density gradient. The enzyme is readily washed out of cells with isotonic NaCl solution. The K_m is 0.6 mM for GTP. The activity is inhibited by 78 ± 12% with the increase in Ca^{2+} concentration from 10^{-9} to 10^{-7} M. GTP hydrolysis is inhibited by the same concentrations of Ca^{2+} which block the sodium conductivity of the rod outer segment cytoplasmic membrane (Hagins, W.A. and Yoshikami, S. (1974) Exp. Eye Res. 18, 299—305).

Introduction

One of the main problems of photoreception, at present, is the elucidation of the mechanism of signal transmission from the light receptor, rhodopsin, which is localized in the disc membranes of photoreceptor cell outer segments, to the cytoplasmic membrane of a photoreceptor cell. Absorption of a quantum of light in a rhodopsin molecule reduces the Na^+ current through the cytoplasmic membrane by 2—3% [1] which in its turn brings about the change of membrane potential. Using the calcium ionophore X537A, Hagins and Yoshikami [2] showed that the increase in intracellular Ca^{2+} concentration from 10^{-9} to

Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

10^{-6} M simulates the light action causing a decrease in the ion current through the cytoplasmic membrane. This result has served as a strong argument in favour of the hypothesis, that bleaching of rhodopsin leads to the appearance, in the rod outer segment cytoplasm, of free Ca^{2+} which, in turn, blocks the sodium conductivity of the cell membrane [3].

There are two possible ways for the Ca^{2+} to affect the sodium conductivity of the cytoplasmic membrane of photoreceptor cells. The first is the direct binding of Ca^{2+} to the components of the cell membrane sodium channel leading to the decrease in conductivity of this structure for Na^+. The second is that Ca^{2+} affects one of the intermediate stages in a hypothetical chain of enzymatic reactions which, ultimately control the conductivity of sodium channels. The first possibility is not likely since there was no observable influence of Ca^{2+} on sodium conductivity of the artificial phospholipid membrane modified by the components of the cytoplasmic membrane of the photoreceptor cell outer segments [4]. This urged us to look for an enzyme system in the rod outer segment which might be efficiently controlled by low concentrations of Ca^{2+}.

The data presented in this paper indicate the presence in the photoreceptor cells outer segments of an enzyme system which is responsible for the GTP hydrolysis and is inhibited by low Ca^{2+} concentrations.

Methods

Isolation of retinal rod outer segments

The rod outer segments were isolated from the retina of frog (*Rana temporaria*) by two methods.

(1) Each dissected retina was placed in a solution containing 115 mM NaCl/2.5 mM KCl/2 mM $MgCl_2$/10 mM Tris-HCl (pH 7.4)/40% (w/v) sucrose (5 ml solution per 1 ml retina). The retina was homogenized for 1 min in the same solution in a cylindrical vessel with the help of a rotating Teflon pestle (300 rev./min) with a gap of 2—3 mm between the pestle and walls of the vessel. The suspension was centrifuged at 15 000 $\times g$ for 10 min.

The supernatant containing the rod outer segment suspension was diluted twice with the same solution which was free from sucrose. The rod outer segments were sedimented (20 000 $\times g$, 15 min). The rod outer segment pellet was then suspended in the appropriate solution.

(2) The retinae were homogenized by the same method in a solution having the same ionic composition and containing 28—29% (w/v) of Ficoll (final concentration). The suspension was placed in a centrifuge tube and layered with the same solution containing 20% Ficoll. Centrifugation was performed for 30 min in a horizontal rotor at 100 000 $\times g$. The rod outer segment layer formed between the two layers of Ficoll was sucked out with a syringe, then suspended in 20% Ficoll, layered on the solution containing 27.5% of Ficoll, and centrifuged under the same conditions. The rod outer segment layer was collected and the suspension was 3 times diluted in the solution free from Ficoll; the rod outer segments were sedimented for 15 min at 20 000 $\times g$ and then resuspended in the appropriate solution. All operations were carried out at 2—4 °C.

Judging by the absorption spectra in the range of cytochrome absorption,

the obtained rod outer segment preparations contained hardly any noticeable impurities of mitochondria. The ratio of absorbances of A_{280}/A_{500} was 2.5—3.0.

Determination of nucleoside triphosphatase activity

The nucleoside triphosphatase activity was determined in the media containing 50 mM Tris-HCl (pH 7.5) 2 mM $MgCl_2$, 2 mM nucleoside triphosphate (ATP or GTP), a rod outer segment preparation (0.3—1.0 mg of protein/ml), 1 μg/ml oligomycin and 0.2 mM ouabain. Before measuring the activity, the rod outer segment preparation was thoroughly homogenized by repetitively running it through a syringe needle with an inner diameter of 0.15 mm. The reaction was carried out at 37°C for 15—40 min in a volume of 0.6 ml and stopped by adding the same volume of 16% trichloroacetic acid. Each determination of the activity was performed twice. The error of the activity measured did not exceed 10%. Every experiment was repeated not less than four times.

Trichloroacetic acid was added into the control sample before the substrate. The activity was determined as the quantity of μmol of inorganic phosphate formed 1 h per mg of protein.

Analytical methods

Inorganic phosphate (P_i) was estimated by the method described by Bonting [5]. The sensitivity of the method is 0.025 μmol of P_i. The products of GTP hydrolysis were analysed by thin-layer chromatography on poly(ethylene imine) cellulose (Merk) according to Bignetti et al. [9] with [^{3}H]GTP (Amersham) 25 Ci/mol as substrate. Protein was determined by the method of Lowry et al. [6].

Fractionation of the rod outer segment preparation

This was carried out in a linear sucrose density gradient (0.75—1.35 M) in a buffer having the saline composition given above. The suspension of the rod outer segment preparation in isotonic solution containing 0.7 M sucrose was layered on the gradient and centrifuged in a horizontal rotor for 1 h at 100 000 × *g*. Fractions were then collected and 0.3 ml of each fraction was added into a sample for measurement of the calcium-inhibited GTPase activity.

Materials

GTP and ATP were obtained from Reanal, Calbiochem and Sigma. Oligomycin, ouabain, EGTA and EDTA were from Sigma. The other reagents were 'chemically pure' and 'extra pure', U.S.S.R. standard.

Results

GTP and ATP hydrolysis

Suspension of rod outer segments homogenised by running them through a syringe needle catalyzes the hydrolysis of GTP at a rate of 1—3 μmol P_i per mg of protein per h. The rate of P_i yield was linear for 40 min under the conditions

of our experiment. Additional destruction of rod outer segments either by freezing-thawing or by treating the rod outer segment suspension with ultrasound does not produce any additional increase in activity. Thus, the indicated treatment of the preparation is sufficient for ensuring maximum accessibility of substrate to enzyme. It should be noted that the activity of preparation exhibits fairly wide variation (3—4-times) from isolation to isolation.

The activity with ATP as substrate is 0.6—0.8 μmol P_i/mg per h, its values varying rather insignificantly with different isolations of the rod outer segment preparation.

The products of reaction are: inorganic phosphate (since the phosphomolibdate method we were using is specific with regard to P_i [7]), GDP, and some quantity of GMP as shown by thin-layer chromatography.

Inhibition by Ca^{2+}

The solutions used in isolating rod outer segments and determining enzyme activity contain impure amounts of Ca^{2+} (approx. 10^{-5} M according to the nominal content of Ca^{2+} admixtures in the commercial reagents used). If the Ca^{2+} concentration is lowered by adding 3.3 mM of EGTA to the solutions, the GTP hydrolysis rate increases 2—3-fold. Conversely, an increase in the Ca^{2+} concentration to 3.3 mM makes the activity fall 1.5—2-fold. In the control experiments, EGTA and Ca^{2+} used in the above indicated concentrations did not influence the results in determining inorganic phosphate by the phosphomolibdate method. The sum total of these data is indicative of the fact that the GTP hydrolysis rate in rod outer segment preparations is controlled by Ca^{2+}.

The maximum rate of GTP hydrolysis observed in our experiments was 7.4 μmol P_i/mg per h (at Ca^{2+} concentration less than 10^{-9} M). The ATP hydrolysis rate in the presence of EGTA and $CaCl_2$ varies insignificantly and the activity in the presence of Ca^{2+} is inhibited by 25 ± 15% with respect to the activity in the presence of EGTA (Table I). Oligomycin and ouabain at the concentrations of 1 μg/ml and 0.2 mM, respectively, inhibit the rate of ATP hydrolysis by 45—

TABLE I

CALCIUM-DEPENDENT HDYROLYSIS OF GTP AND ATP BY THE ROD OUTER SEGMENT SUSPENSION AND BY SOLUBLE AND MEMBRANE FRACTIONS OF ROD OUTER SEGMENTS (ROS)

The rod outer segment pellet resuspended in 1 mM Tris-HCl, pH 7.5, homogenized, the soluble and membrane fractions separated by centrifuging the preparation for 20 min at 25 000 × *g*. For conditions of determining the activity see Methods. In each individual experiment the activity of every fraction in the presence of 3.3 mM EGTA or Ca^{2+} (3.3 mM $CaCl_2$) was compared with the activity of rod outer segment homogenate, which was taken to be 100 units. The given activity values are the average values obtained in seven experiments ± S.E.

	Activity (arbitrary units)			
	with GTP		with ATP	
	EGTA	$CaCl_2$	EGTA	$CaCl_2$
ROS (hypotonic shock)	100	22 ± 12	100	75 ± 15
Supernatant 250 000 × *g*	380 ± 45	42 ± 14	83 ± 7	75 ± 13
Pellet 25 000 × *g*	49 ± 6	50 ± 11	123 ± 12	118 ± 12

55%. These inhibitors diminish the rate of GTP hydrolysis by 5—12%, both at high and low concentrations of Ca^{2+}, without changing the rate of calcium-dependent GTP hydrolysis.

The substrate specificity of calcium-dependent nucleoside triphosphatase activity is revealed more explicitly when the soluble and membrane fractions of the rod outer segment preparation are separated. GTPase activity of the soluble fraction obtained after the hypotonic shock of rod outer segments is 4-times higher than that of the initial preparation, while it is almost completely suppressed by Ca^{2+}. Conversely, ATP hydrolysis is lower in the soluble fraction than in the membrane one (Table I). Moreover, the degree the Ca^{2+} inhibits ATP hydrolysis by soluble fraction is substantially smaller than in the case of GTP.

Fig. 1 shows the dependence of GTPase activity of the homogenated rod outer segment suspension on GTP concentration for high and low concentrations of Ca^{2+}. For $pCa^{2+} = 9$, the dependence is described by the Michaelis equation. The apparent K_m is 0.6 mM. In the presence of Ca^{2+} the activity does not depend on the GTP concentration (in the range of GTP concentration from 10^{-4} to $3 \cdot 10^{-3}$ M).

Adding EDTA in 3 mM concentration to the reaction media reduces the activity to zero. From the results given in Table II it is inferred that the calcium-inhibited GTP hydrolysis is specifically activated by Mg^{2+}, the maximum activity being achieved with an Mg^{2+} concentration equal to that of the substrate.

Incomplete suppresion of GTP hydrolysis by Ca^{2+} in the rod outer segment preparation maybe due to at least two factors. On the one hand, it may be connected with the fact that a relevant enzyme is capable of hydrolyzing GTP even in the presence of large Ca^{2+} concentrations. It is possible, on the other hand, that in the case under discussion we are dealing with a number of

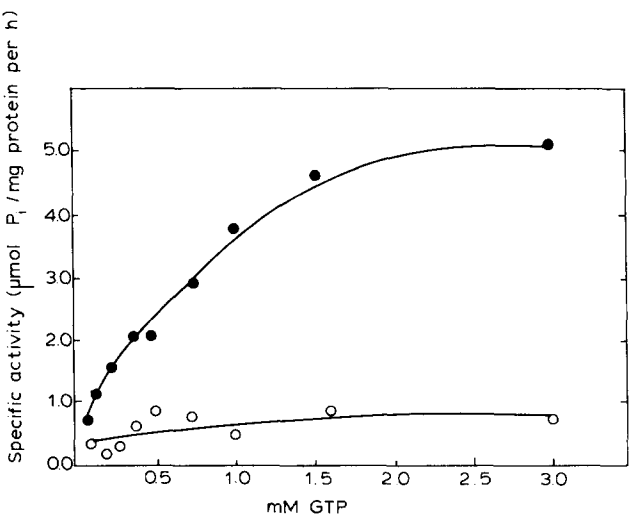

Fig. 1. Relation between the rate of GTP hydrolysis by rod outer segment suspension and GTP concentration. For reaction conditions see Methods, ●———●, with 10^{-9} M Ca^{2+} in media; △———△, with 3.3 mM $CaCl_2$ in media. Rod outer segments isolated in sucrose (see Methods) and homogenized by passing through a syringe needle.

TABLE II

EFFECT OF DIFFERENT DIVALENT IONS ON CALCIUM-DEPENDENT GTP HYDROLYSIS BY THE ROD OUTER SEGMENT HOMOGENATE

The conditions of determination of the activity are as described in Methods, except for the concentration of the bivalent metal ions the values of which are given in the first column of the Table.

Metal (mM)	Activity (arbitrary units)	
	3.3 mM EGTA	3.3 mM Ca^{2+}
None (3 mM EDTA)	0	0
0.1 Mg^{2+}	8	—
1 Mg^{2+}	75	—
2 Mg^{1+}	100	20
10 Mg^{2+}	100	—
2 Cu^{2+}	12	20
2 Mn^{2+}	20	20
2 Co^{2+}	12	15
2 Sr^{2+}	15	20
2 Zn^{2+}	18	30

enzymes, the activity of one of them being suppressed by Ca^{2+}, while the activity of others is independent of Ca^{2+}. The following facts testify in favour of the second hypothesis: (1) the increase in the rate of GTP hydrolysis by the soluble fraction of rod outer segments is accompanied by heightening of the degree of Ca^{2+} inhibition; (2) all the ions tested, except Mg^{2+}, restore the GTP hydrolysis rate but only to a level near that observable after maximum suppression of Mg^{2+}-dependent activity by Ca^{2+}.

Further experiments were devoted to establishing the range of Ca^{2+} concentrations in which the inhibition of GTPase activity is taking place. Solutions with specified Ca^{2+} concentrations were prepared by titrating 0.2 mM $CaCl_2$ solution with EGTA solution. Ca^{2+} concentration was calculated by assuming the value of dissociation constant of EGTA-Ca complex, pH 7.5 (K_{eff}), to be equal to $8.3 \cdot 10^{-8}$ M [8]. In order to compare our results with the data obtained by Hagins and Yoshikami [2], we titrated GTPase activity with calcium under the same conditions as used by these authors, using their value of $K_{eff} = 10^{-7.6}$ for pH 7.15.

Fig. 2 shows the rate of GTP hydrolysis by rod outer segment suspension as a function of Ca^{2+} concentration in the reaction media for pH 7.5 and 7.15. It is seen from Fig. 2 that the major part of activity is inhibited as the Ca^{2+} concentration is increased from 10^{-9} to 10^{-7} M. An increase in Ca^{2+} concentration from 10^{-7} to 10^{-5} M produces no significant change in the activity, while in the range of 10^{-5}—10^{-4} M Ca^{2+} the activity falls to its minimal values. The differences observed for different pH values virtually amounted to the changes in the plateau level at pCa^{2+} (5—7).

Determination of GTP activity of rod outer segment preparations, and of the degree to which it is inhibited by Ca^{2+}, was carried out with unbleached preparations in darkness as well as with preparations containing 10—100% of bleached rhodopsin. There were no noticeable differences in the activity.

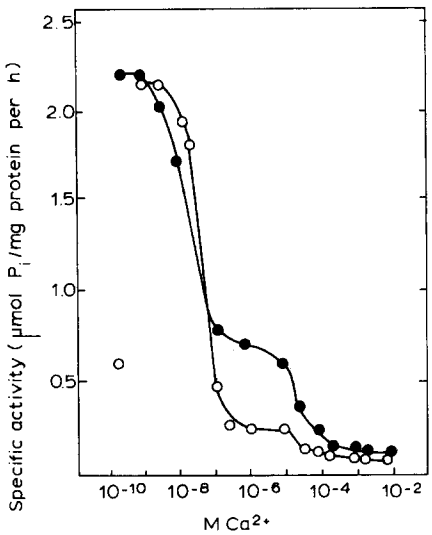

Fig. 2. Relation between the rate of GTP hydrolysis by rod outer segment suspension and Ca^{2+} concentration. For reaction conditions see Methods. ●———●, pH 7.15; ○———○, pH 7.5.

Localization of GTPase activity

To determine whether the GTPase activity is associated with the outer segments of photoreceptor cells, we fractionated the preparations of rod outer segments in the sucrose density gradient (see Methods). In order to reduce the portion of broken outer segments in the preparation, the usual homogenizing technique was replaced by gentle stirring of the retinal suspension. The rod outer segment preparation visually checked was seen to contain 60—80% of intact outer segments. It is apparent from Fig. 3A that the distribution of GTPase activity coincides with the distribution of rhodopsin, which is indicative of the GTPase localization in the outer segments of photoreceptors. At the same time, a significant part of the activity is in that volume of 0.7 M sucrose in which the preparation under study was initially applied onto the gradient. Obviously, this activity is related to extracellular soluble protein in the preparation of the rod outer segment suspension. Its presence in the low density fraction might be explained by assuming that the GTPase of outer segments is a soluble protein and is readily washed out of outer segments when they are broken or disrupted. To verify this possibility, the segments of the same preparation which had been used in the previous experiments (Fig. 3A) were thoroughly disrupted by running them through a syringe needle and then fractionated in the sucrose density gradient (Fig. 3B). This proved that only a small part of the activity is found at the peak of outer segments, while the main part of GTPase is washed out of cells and remains in the volume containing soluble proteins.

Intracellular localization of calcium-inhibited GTPase

As noted in the preceding section, the calcium-inhibited GTPase from rod outer segments of the retina is most probably a soluble enzyme. This suggestion

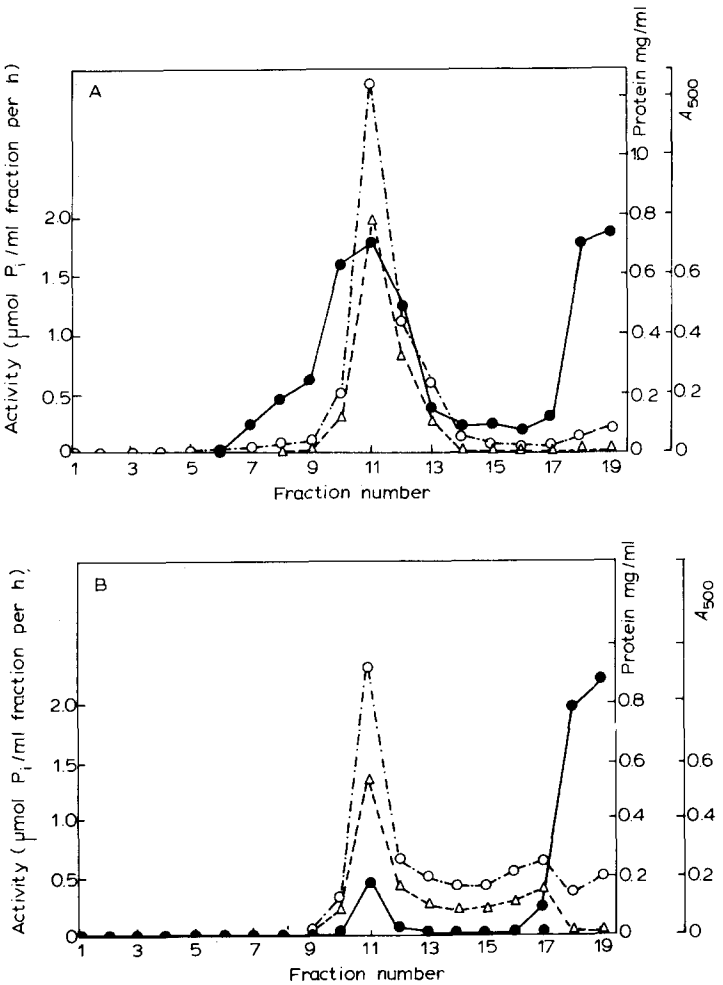

Fig. 3. Fractionation of rod outer segment preparation in sucrose density gradient. Fraction volume is 2 ml. Measured in each fraction were: absorbance of rhodopsin at 500 nm (△- - - - -△), protein concentration (○· — · — ·○), and GTP hydrolysis rate (●———●) A, 'intact' segments isolated in mild conditions; B, thoroughly homogenized segments. For conditions of experiment see Methods and elsewhere in the text.

is also supported by the data given in Table I.

Nevertheless, we carried out the following experiments on washing soluble proteins out of broken rod outer segments by isotonic solution since hypotonic solutions used in the experiments are capable of washing out the membrane proteins.

After homogenization, a total amount of Ca-inhibited activity ($A_t = A_{sp} \times$ volume $\times$ protein concentration) in the sample was determined, then the membrane and soluble fractions were separated (30 min, 150 000 $\times g$), and the total activities of sediment and supernatant were determined. After that the sediment was resuspended and the procedure was repeated twice. Even the first washing releases 90% of activity into the soluble fraction (Fig. 4). The two

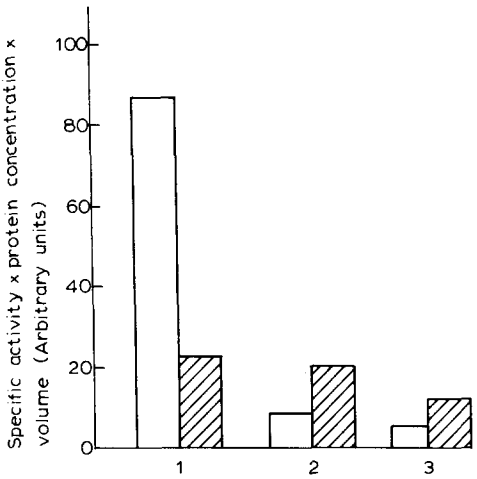

Fig. 4. Washing of rod outer segment suspension by isotonic solution. Total activity of supernatant (clear columns) and pellet (hatched columns) was determined after homogenizing and centrifuging rod outer segments at 150 000 × *g*. Abscissa show the number of washings. 100 units correspond to the total activity of the initial rod outer segment homogenate.

succeeding washings release only a small wash-out of activity. Even after three washings, the membrane fraction retains a small Ca-inhibited GTPase activity. It should be noted that, in all the experiments on separation of the membrane and soluble fractions of rod outer segments, the sum of the activities of the two fractions is 20—50% higher than the total activity of the initial rod outer segment homogenate. Unlike GTPase activity, 80—90% of ATPase activity remains to be bound with the particulate fraction, following double washing of the rod outer segment homogenate with isotonic solution.

These results point to the fact that the GTPase under study is a soluble protein, and consequently occurs free in the cytoplasm of retinal rod outer segments. At the same time, we cannot rule out the possibility that during experimental manipulations, there were some factors aiding the transition of the extrinsic-membrane proteins into solution. One such factor may be sucrose [9] in the concentrated solution used during the isolation of outer segments. However, the experiments carried out on the rod outer segment preparations isolated in Ficoll (see Methods) showed that in this case, as well, the GTPase activity is readily washed out by isotonic solution. Another factor which, in principle, might cause dissociation of near-membrane proteins is calcium. Its concentration in rod outer segments is low [2]. At the same time in the working solution it was about 10^{-5} M and capable of changing the enzyme-membrane link. To eliminate this possibility, we carried out the rod outer segment isolation in a non-calcium media ($pCa^{2+} = 9$). In these conditions the activity was also found to be readily washed out of cells.

Discussion

The results of experiments by Caretta and Cavaggioni [10], Robinson and Hagins [11] and De Azeredo et al. [12] testify that the GTP concentration in

the retinal rod outer segments exceeds 1 mM. These results allow for the assumption that there are in vivo conditions capable of ensuring high activity of calcium-inhibited GTPase which we discovered and which are characterized by rather low affinity for the substrate (K_m = 0.6 mM).

The results which made us call the activity under study 'GTPase' are: (1) the hydrolysis rate of 2 mM GTP by rod outer segment suspension, at $pCa^{2+} = 9$, is 5—7-times higher than that of ATP under the same conditions; (2) calcium inhibits hydrolysis of GTP to a greater degree than it inhibits that of ATP (Table I) (3) Ca-inhibited GTPase activity is very easily washed out of cells (Fig. 4), whereas 80—90% of ATPase activity is linked mainly with the membrane fraction and is not inhibited by calcium (Table I). Since the activity of preparations was determined in the presence of ouabain and oligomycin, the inhibitors of $(Na^+ + K^+)$-ATPase and mitochondrial ATPase, respectively and Ca-inhibited ATPase activity in the soluble fraction can be explained either by the presence of some amounts of Mg^{2+}-ATPase [13], or by the ability of GTPase to weakly hydrolyze ATP. A final conclusion concerning the substrate specificity of enzyme can be reached only after it is purified. In principle, the GTPase activity could be copied by a GTP-dependent protein kinase, a protein which phosphorylates, and phosphoprotein phosphatase that may break down GTP into GDP and P_i. However we failed to find it in the rod outer segment preparations of such highly active calcium-dependent protein kinase system [14].

The procedure of rod outer segment isolation is inevitably linked with partial disruption of cells. This leads to an uncontrollable wash-out of soluble GTPase (60—90% of GTPase activity is left in sucrose solution in the process of rod outer segment isolation) and to a considerable scatter in the values of specific activity of purified preparations of retinal rod outer segments. Since there is no suggestion that this activity occurs in other retinal cells, it is difficult to evaluate the GTPase activity in native rod outer segments.

Most of the GTPase activity is inhibited by increasing the Ca^{2+} concentration from 10^{-9} M to 10^{-7} M (Fig. 2). This range coincides with the ranges of Ca^{2+} concentrations which block sodium conductivity of cytoplasmic rod outer segment membrane in the experiments of Hagins and Yoshikami [2].

The two-step character of the curve of GTPase inhibition by Ca^{2+} may be explained by the presence of two enzymes. Alternatively there may be two or more Ca^{2+}-binding centres in one enzyme with different affinity for the Ca^{2+}. These two possibilities will be resolved only after the pure enzyme is isolated.

Wheeler et al. [15] in 1977, and Bignetti et al. [9] somewhat later, reported on the GTPase activity they found in preparations of retinal rod outer segments. The GTPase of our investigations differs from that previously described [9,15], in at least four properties: (1) its K_m is about 1000-times greater (2) its maximum GTP hydrolysis rate is, at least 10-times higher (3) its activity does not depend on whether rhodopsin is bleached or not and (4) it is effectively inhibited by low concentrations of Ca^{2+}, whereas, according to Bignetti et al. [9], the activity of light-dependent GTPase does not change in the presence of Ca^{2+}.

Wheeler et al. [15] noted the occurrence of the light-independent high K_m (0.1 mM) GTPase activity in rod outer segment preparations. Differences in

kinetic parameters of light-independent GTP hydrolysis between our data and those of Wheeler et al. are, probably explained by high concentrations of Ca^{2+} (10^{-5} M) in solutions with which the authors [15] worked.

Recently, Tacher [16] reported on an ATPase activity in rod outer segment preparation which is similar in certain properties to the GTPase activity described in the present paper. The reasons for some differences between our data and those of Tacher are not yet clear.

The results given in this paper do not enable us to ascribe a definite role in the functioning of rod outer segments to calcium-inhibited GTPase. At the same time, in considering various schemes for its participation in the response of a photoreceptor cell, the following has to be taken into account. The range of Ca^{2+} concentration in which GTPase inhibition takes place coincides with that in which the blocking of sodium channels of cytoplasmic membrane of rod outer segment is observed [2]. This suggests that the ionic conductivity of rod outer segment cytoplasmic membrane is controlled by the activity of calcium-dependent GTPase. This suggestion would be much more plausible if GTPase were structurally associated with the cytoplasmic membrane. Its solubility casts doubts upon the possibility of a direct involvement of GTPase in the regulation of sodium conductivity of the outer segment membrane. In principle, however, it is possible that GTPase has an indirect influence on cytoplasmic membrane conductivity, possibly, through the cyclic GMP system [17,18]. Wheeler and Bitensky [19] demonstrated the possibility of allosteric regulation of cyclic GMP phosphodiesterase by photoactivated GTPase. Goridis et al. [17], on the other hand, showed that the base (dark) level of cyclic GMP phosphodiesterase activity is controlled by low concentrations of Ca^{2+}. It is possible, that the control of the base level of phosphodiesterase activity, as well as the control by light, is effected by means of GTPase.

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