Adenylate kinase activity in rod outer segments of bovine retina

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Abstract

The rod outer segments of bovine retina contain two different adenylate kinases: a soluble activity, which is not sensitive to calcium ion, and an activity bound to disk membranes, which is dependent on the calcium levels. In fact, the maximal activity associated to the disks is reached at Ca\(^{2+}\) concentrations between 10\(^{-6}\) and 10\(^{-7}\) M, which is the range of calcium level actually present in the rod cell. The Michaelis-Menten kinetics of the enzyme activity on disk membranes was determined and the actual concentrations of ATP, AMP and ADP were measured in the photoreceptor outer segment. Therefore, the physiological relevance of the adenylate kinase activity was discussed considering the above results. The formation of ATP catalyzed by the enzyme seems appropriate to supply at least some of the reactions necessary for phototransduction, indicating that ATP could be regenerated from ADP directly on the disk membranes where the photoreception events take place. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The problem of availability of ATP in the rod outer segment (ROS) during photoreception remains to be elucidated. The regeneration of ATP is likely to happen in the ROS, as close as possible to the disk membranes where the main reactions for the phototransduction need an immediate source of energy. The finding that glyceraldehyde-3-phosphate dehydrogenase is present in large quantities in ROS suggests that part of the ATP and GTP synthesis takes place from glycolysis [1] which produces about 95 \(\mu\)M ATP/s [2]. One of the major needs of ATP for phototransduction reactions is the synthesis of cGMP. In the dark, a value of 28 \(\mu\)M ATP/s was obtained for the basal cGMP turnover [3]. Following illumination, the cost of synthesizing cGMP increases by a factor of 4.5 [3] reaching about 126 \(\mu\)M ATP/s, which is higher than the rate of ATP synthesized by glycolysis. Additional energy was shown to come from the system of creatine kinase with diffusive metabolites such as phosphocreatine and creatine, capable of maintaining a high local ATP/ADP ratio [4]. However, the energy needed for phototransduction reactions such as the activation of transducin and the phosphorylation of rhodopsin, should be supplied within milliseconds. For this reason, the source of energy must be located on the disk membranes in order to avoid the slow diffusion of energy metabolites.
In this paper results are shown which indicate that ROS of bovine retina also contain an adenylate kinase activity. The data suggest that at least two different adenylate kinases are present in ROS homogenates: a soluble activity and an activity bound to the disk membranes. The latter accounts for a rate of 160 \( \mu \text{M ATP/s} \) at physiological ADP concentrations, which is of the order of magnitude of the amount of ATP which has to be regenerated from ADP to support those phototransduction events which take place on the disk membranes.

2. Materials and methods

2.1. Osmotically intact ROS and disk preparations

In a typical experiment rod outer segments were isolated from 30 bovine retinas in dim red light following the method of Schnetkamp and Daemen [5] by sucrose gradient centrifugation. Osmotically intact disks were obtained after bursting ROS for 3 h in 30 ml of 5% Ficoll (Sigma) in distilled water containing 5 mM DTT and 70 mg/ml leupeptin and then by collecting them in dim red light at the 5% Ficoll surface after centrifugation for 2 h at 25 000 rpm in a Beckman FW-27 rotor [6]. ROS or disk preparations were stored in the dark at \(-80^\circ\text{C}\).

2.2. ROS fraction preparation

Intact ROS were first washed in an isotonic medium containing 40 mM Tris-maleate pH 7, 120 mM KCl and 10% sucrose. Washed ROS were centrifuged for 10 min at 1500 \( \times \) g and the pellet resuspended in distilled water and then homogenized in a Potter apparatus for 10 min at ice temperature. Homogenated ROS were then centrifuged for 15 min at 1500 \( \times \) g. The supernatant was withdrawn and the pellet washed twice and centrifuged again. The pellet was then resuspended in the initial volume of distilled water and the second supernatant was added to the first.

2.3. ATP and AMP formation

ATP and AMP formation were measured in daylight on purified ROS or disks in a reaction mixture containing 40 mM Tris-maleate pH 7, 120 mM KCl, 5 mM MgCl\(_2\); 2.5 mM ADP and 1.2 mg of ROS or disk protein were added to a final volume of 1 ml. Free Ca\(^{2+}\) concentrations of the mixture were obtained with a Ca\(^{2+}\)-EGTA buffer [7] (EGTA was 2 mM). In order to avoid the contribution for ATP synthesis or hydrolysis by disk Ca\(^{2+}\)-ATPase [8], all the samples were incubated in the presence of 1 \( \mu \text{M} \) thapsigargin and 10 mM vanadate. Samples were kept at room temperature (20\(^\circ\)C). At appropriate intervals, 0.1 ml aliquots of the reaction mixture were withdrawn and added to 50 \( \mu \text{l} \) of 25% perchloric acid. Samples were centrifuged for 15 min at 1500 \( \times \) g, then 0.1 ml of supernatant was withdrawn, neutralized with K\(_2\)CO\(_3\) and centrifuged again to remove potassium perchlorate. 0.05 ml aliquots of each neutralized perchloric extract were used for the assay of ATP or AMP.

2.4. ATP and AMP assay

ATP or AMP was assayed enzymatically, following the methods of Bârzu and Michelson [9] with minor modifications and Kornberg and Pricer [10] respectively. For ATP assay, the medium contained 0.1 ml of neutralized perchloric extract, 50 mM Tris-HCl, pH 8.0, 1 mM NADP, 10 mM MgCl\(_2\), 5 mM glucose in 1 ml final volume. Samples were analyzed spectrophotometrically before and after the addition of 4 \( \mu \text{g} \) of purified hexokinase/glucose-6-phosphate dehydrogenase (Boehringer). The rise in absorbance at 340 nm was proportional to the ATP concentration. For AMP assay, 0.05 ml of neutralized perchloric extract was added to an assay medium containing 50 mM Tris-HCl, pH 8.0, 200 \( \mu \text{M} \) NADH, 10 mM MgCl\(_2\), 1 mM phosphoenolpyruvate, 10 \( \mu \text{g} \) of purified pyruvate kinase/lactate dehydrogenase mixture. The decrease in absorbance (due to the NADH oxidation), proportional to the AMP concentration, was measured after the addition of 4 \( \mu \text{g} \) of purified adenylate kinase (Boehringer) to the mixture.

Protein concentrations were determined using the Bradford method [11].

2.5. Endogenous ATP, ADP and AMP estimate

Purified ROS prepared in dim red light were pro-
cessed in the dark or in daylight (1200 W) by adding 360 μl of 25% perchloric acid. The samples were centrifuged, then supernatants were neutralized with K₂CO₃ and centrifuged again to remove potassium perchlorate. Aliquots of 50 μl were charged on a Source 15Q FPLC (60×6 mm) column (Pharmacia) and separated with a linear gradient of KCl in phosphate buffer (buffer A was 0.012 M KCl and KH₂PO₄ at pH 4; buffer B was 0.250 M KCl and KH₂PO₄ at pH 5) at a flow of 2 ml/min. Single endogenous nucleotide peaks were separated and areas integrated. To calculate total nmoles of ATP, ADP and AMP, the results of integrations were compared with a calibration curve built in the same conditions with standard solutions of nucleotides. The concentration of rhodopsin brought in solution with 2% octyl glucoside was determined spectrophotometrically in each sample. The concentrations of the endogenous nucleotides obtained by FPLC were calculated by comparison with rhodopsin concentration on the assumption that rhodopsin in intact ROS is about 3 mM [12].

3. Results

To measure adenylate kinase activity, homogenated ROS or isolated disk membranes were incubated in the light and in the presence of 2.5 mM ADP at an external Ca²⁺ concentration of 1 μM (see Section 2). Fig. 1 shows ATP (or AMP) production by homogenated ROS or disk membranes. A value of approx. 150 nmoles ATP/mg protein/min can be deduced from the first minutes from Fig. 1 for homogenated ROS preparations and a value of about 80 nmoles ATP/mg protein/min for purified disk membranes.

Table 1

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Homogenated ROS</th>
<th>Supernatant from homogenated ROS</th>
<th>Pellet from homogenated ROS</th>
<th>Ficoll-purified disk membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total activity (IU; nmoles ATP/ml/min)</td>
<td>2242 ± 150</td>
<td>1548 ± 100</td>
<td>546 ± 40</td>
<td>319 ± 20</td>
</tr>
<tr>
<td>Protein concentration (mg/ml)</td>
<td>16 ± 2</td>
<td>4 ± 0.5</td>
<td>13 ± 1.5</td>
<td>4 ± 0.5</td>
</tr>
<tr>
<td>nmoles ATP produced/min/mg protein</td>
<td>140 ± 27</td>
<td>387 ± 73</td>
<td>42 ± 8</td>
<td>80 ± 15</td>
</tr>
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</table>

External calcium ion concentration was 1 μM. Total activity is expressed in IU/ml (nmoles of ATP produced/min in 1 ml of homogenated ROS). For disk membranes, adenylate kinase activity is referred to 1 ml of homogenated ROS. Each figure represents the mean ± S.D. of four measurements.
duced/min/mg of protein increases by a factor of 2 in the purified disk preparation with respect to that of the homogenate pellet.

In order to investigate the sensitivity of adenylate kinase to calcium, experiments were performed at different external concentrations of free calcium ions. Fig. 2 shows that the adenylate kinase activity of both homogenated ROS and disk membranes seems sensitive to free calcium concentration in a similar way, with a $K_{1/2}$ for calcium of about $5 \times 10^{-6}$ M. On the contrary, the enzyme activity of the soluble fraction of homogenated ROS maintained a constant value within the experimental errors (about 370 nmoles ATP produced/min/mg of protein), showing no sensitivity to free calcium ion concentrations.

The adenylate kinase activity on the disk membranes was measured as a function of ADP concentration and the resulting Michaelis-Menten kinetics is shown in Fig. 3. The Lineweaver-Burk plot of the data gave a $K_m = 1.48$ mM and $V_{max} = 155$ nmoles ATP/min/mg protein.

The actual concentrations of ADP, ATP and AMP in vivo were measured in homogenated ROS by means of FPLC. Assuming a value of 3 mM for the concentration of rhodopsin in intact ROS [12], the results are shown in Table 2.

Surprisingly, there are no striking differences between dark and illuminated samples, suggesting that the ATP consumed by the phototransduction events is rapidly replaced by the increase in overall nucleotide turnover. The $K = [ADP]^2/[ATP][AMP]$ calculated for the concentrations of the nucleotides in ROS kept in the dark gave a value of 2.296, which is very close to the value of 2.260 for $K$ at equilibrium at 25°C and pH 7.4 [14]. This suggests that the concentration of the adenylate kinase in ROS is adequate to maintain the equilibrium between AMP, ATP and ADP and the metabolic pathway is not blocked. Instead, the apparent deviation from equilibrium found in the light ($K = 1.45$) could indicate that in the light the adenylate kinase activity is limiting.

### Table 2

<table>
<thead>
<tr>
<th>AMP, ADP and ATP content in ROS of bovine retina</th>
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<tbody>
<tr>
<td>Rod outer segment</td>
</tr>
<tr>
<td>Light</td>
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<tr>
<td>Dark</td>
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</tbody>
</table>

The concentrations of the endogenous nucleotides separated by FPLC were calculated by comparison with rhodopsin on the assumption that rhodopsin concentration in intact ROS is about 3 mM [12].
4. Discussion

The results of the present study strongly suggest that the adenylate kinase activity in ROS belongs to two different isoenzymes: a soluble one, which is not sensitive to calcium ion concentration, and another, bound to disk membranes, which is instead sensitive to calcium ions. This calcium sensitivity is probably mediated by a cofactor bound to the membrane surface and effective only on membrane associated adenylate kinase.

The presence in purified ROS of a soluble adenylate kinase similar to the well known cytoplasmic isoenzyme [15–17] was previously reported by Hall and Kühn [18], while examples of membrane-bound adenylate kinases were already shown by different authors in *Rhodobacter sphaeroides* chromatophores [19], and in vertebrate brain synaptosomes [20,21].

From the Michaelis-Menten kinetics of Fig. 3, the adenylate kinase activity bound to the disks has a rate of about 70 nmoles ATP/min/mg protein at an ADP concentration of 1.3 mM, the endogenous content found in ROS (see Table 2). This means a value of about 160 W M ATP/s assuming that rhodopsin (molecular mass 39 kDa), present in ROS at a concentration of 3 mM [12], constitutes about 85% of the total disk protein. The in vivo rate of adenylate kinase activity is probably lower than that measured in vitro due to the presence of AMP and ATP. In fact, an attempt to calculate the rate due to competitive product inhibition gave values of about 30–40 nmoles of ATP/min/mg protein, using $K_m$ (ATP) = 0.3 mM and $K_m$ (AMP) = 0.5 mM found in the literature [14]. On the other hand, local nucleotide concentrations near the disk membranes may be different from the total values shown in Table 2, as could happen for the ATP produced which is probably rapidly consumed and therefore subtracted from equilibrium. Anyway, a rate of ATP production of 40 nmoles of ATP/min/mg protein would also be appropriate for supplying some of the reactions necessary for phototransduction which take place on the disk membranes. In fact, the phosphorylation of the photoactivated rhodopsin seems to need about 40 nmoles ATP/min/mg protein [22] which makes 90 µM ATPs. The replenishment of GTP hydrolyzed by transducin has not yet been calculated, although it is quite probable that it needs less ATP than rhodopsin phosphorylation.

Another physiological important point is that the ATP production by adenylate kinase is fully activated at Ca$^{2+}$ concentrations between $10^{-6}$ and $10^{-7}$ M (see Fig. 2), which is the range of calcium level actually present in the rod cell. The decrease of the internal Ca$^{2+}$ concentration from 0.5 µM to 0.1 µM following illumination of ROS [23] does not seem to affect the adenylate kinase activity.

The advantage of the presence of an adenylate kinase on the ROS disks follows from the fact that ATP is regenerated from ADP in situ directly on the disk membranes where the main reactions for the photoreception need an immediate source of energy. In this way any low diffusion of metabolites or ATP molecules from ROS cytoplasm is avoided and phototransductive processes can rapidly take place on the disk surface.

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References