Ca-ATPase Activity in the Rabbit and Bovine Lens

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Membrane-rich vesicle preparations of rabbit and bovine lenses were prepared in such a manner as to preserve ATPase activity. The lipid:protein ratio of these preparations was increased 22- to 33-fold with a 94% recovery of total phospholipid. Using this preparation, calcium stimulated ATPase was routinely determined in both individual lenses and in pooled specimens. The pattern of stimulation of ATPase activity by a range of calcium concentrations was found to be similar in membrane preparations of epithelium and cortex, from rabbit and bovine lenses. The concentration of calcium necessary for half-maximal stimulation of ATPase activity was approximately $10^{-6}$ M. Calcium concentrations in excess of $10^{-4}$ M reduced the ATPase activity. Calcium-ATPase was undetectable in the lens nuclear region of both species. The regional distribution of sodium-potassium ATPase was also measured. Invest Ophthalmol Vis Sci 29:982–987, 1988

The concentration of freely diffusible calcium in the lens cytoplasm is 100-1000 times lower than the level in the surrounding aqueous humor. In 70% of human cataracts the intracellular calcium and sodium concentrations in the lenses are dramatically elevated. While deranged monovalent cation concentrations in the lens are recognized to be deleterious because they cause osmotic swelling, the effect of elevated lenticular calcium levels is no less harmful to lens function. Calcium may cause lens opacity in a number of ways, for example, by interacting with cytoplasmic proteins, or by activating proteases or lipases in the lens.

It is probable that calcium leaks into the lens, and the inward leak is balanced by an active extrusion mechanism, most likely a calcium-stimulated ATPase. There is evidence for calcium-stimulated ATPase activity in the lens. Hightower et al showed Ca-ATPase activity to be prominent in the lens epithelium, but low or nonexistent in the lens cortex and nucleus. The detection of membrane Ca-ATPase in the lens cortex and nucleus homogenates is complicated by the large amount of nonmembrane crystallin proteins present which can interfere with the activation of Ca-ATPase and its detection.

To enhance the detection of Ca-ATPase we prepared a membrane-enriched sample from lens epithelium, cortex and nucleus. The methodology employed was the same as that routinely used in making membrane-enriched preparation of cardiac sarcoplasmic reticulum containing Ca-ATPase. This procedure was used because commonly used lens membrane preparations employ chaotropic agents to solubilize cytosolic lens crystallins. These chaotropic agents would be expected to abolish Ca-ATPase activity.

Materials and Methods

Chemicals

Adenosine 5'-triphosphate as a triethylammonium salt labelled with phosphorous-32 in the terminal phosphate group was purchased from Amersham (Arlington Heights, IL), and Budget-Solve HFP scintillant was purchased from Research Products International Corp. (Mount Prospect, IL). All other reagents were purchased from Sigma Chemical Company (St. Louis, MO).

Animal Tissues

Bovine eyes were obtained fresh from the slaughterhouse from 18-month-old cows. Enucleated globes were kept on ice for at most 2 hr prior to lens extraction. Rabbit eyes were obtained from healthy 2 kg New Zealand strain albino rabbits about 10 weeks old, killed painlessly by intravenous administration of T-61 euthanasia solution (American Hoechst Corp., Somerville, NJ) and the eyes enucleated. The experiments carried out here adhered to the ARVO
Resolution on the Use of Animals in Research. The lens was removed by a posterior approach to the globe and blotted on filter paper moistened with calcium-free saline to remove vitreous and remaining zonules. An epithelial preparation, consisting of the lens epithelium and capsule, was removed from lens by making an equatorial incision through the lens capsule and peeling the capsule together with the adhering epithelial layer away from the cortex. The cortex was separated from the dense nucleus using fine forceps and a sterile scalpel blade.

Membrane-Enriched Microsome Preparation

Cortical, nuclear, and epithelial material was pooled to obtain sufficient material for careful characterization. Pooled material from ten bovine lenses or 26 rabbit lenses, or material from individual lenses was weighed and suspended in six volumes of buffer A containing: 0.3 M sucrose, 10 mM HEPES-HCl pH 7.4, and 2 mM dithiothreitol. Buffer A was prepared fresh on the day of the preparation and kept chilled. The pooled material was homogenized with five to eight strokes of a tight-fitting teflon douncer jacketed in ice. The homogenate was centrifuged at 85,000 g for 75 min. The supernatant was removed for analysis, and the pellet was resuspended in the original volume of buffer A containing 0.6 M KCl using four strokes of the teflon douncer. The pellet was centrifuged again at 85,000 g for 75 min. The supernatant was removed for analysis and the pellet was resuspended in the original volume of buffer A. After centrifuging at 85,000 g for 75 min, the pellet was suspended in buffer A using four strokes of a teflon-coated douncer at a protein concentration of 2–7 mg/ml. The samples were divided into 0.5 ml aliquots and stored frozen at −70°C; the Ca-ATPase activity did not diminish noticeably over a 1-month period. Ca-ATPase activity measured after freezing the sample was comparable to activity measured on unfrozen aliquots.

Determination of Lipid and Protein Content

Protein was determined by the Peterson modification of the Lowry assay. This method was shown by infrared spectroscopy to be more accurate for measuring membrane protein than the Lowry method. Protein concentration for each sample was determined at four dilutions, at least three times, with a precision of ±10.8%.

Phospholipid was extracted from 0.5 ml of the final pooled cortical and nuclear and 0.1 ml of pooled epithelial membrane-enriched suspension using the method of Zigman et al. Lipid extract volumes of 5, 10, 20, and 50 μl containing 28 ng to 1 μg of phosphorus, depending on the sample, were dried under nitrogen. Lipid phosphorus concentration was determined at least three times by the modification of the Bartlett assay, scaled down by a factor of 0.3. A820/μl (correlation coefficients averaged 0.98 ± 0.03) were determined by linear regression and compared to the A820/μg P of standard. Because of the small sample size, the relative standard errors averaged ±50%.

ATPase Activity

Ca-ATPase activity was measured at 37°C in 350 μl of a reaction mixture containing 50 mM histidine- HCl, pH 7.4, 100 mM KCl, 3 mM MgCl2, 5 mM NaNO3, 3 μg/ml ionophore A2317, 0.5 mM [γ32P]-ATP (109 cpm/μmol), microsomes (0.1 mg/ml protein), and either 5 mM EGTA (buffered with tris pH 7.4), or CaCl2-EGTA buffer, prepared as described previously, to give a final CaCl2 concentration of 125 μM. A value of 106 M−1 was used for the apparent binding constant of calcium to EGTA for comparative purposes since this constant and the same buffer equations were used for the characterization of the Ca-ATPase in cardiac SR. Recalculation of free calcium concentration using other equations and binding constants did not alter the shape of our calcium stimulation curve but simply shifted the curve to the left or the right. Calcium and magnesium binding to ATP as well as the effect of pH were considered in calculating the free calcium concentration. pH was kept constant ±0.03 throughout the assay and CaCl2-EGTA buffer regime. Reactions were started with labeled [γ32P]ATP after a 5 min temperature equilibration. ATP hydrolysis was terminated at 10, 20, and 30 min by aliquoting 100 μl of sample into 50 μl ice cold trichloroacetic acid (5% final concentration). 32P liberation was determined by a method based on the extraction of a phosphomolybate complex in isobutyl-alcohol. One hundred microliters of 5% ammonium molybdate in 2.5 N H2SO4 was added to all samples and vortexed simultaneously for 1 min. A 600 μl volume of ice-cold isobutyl-alcohol:benzene, 1:1 (v:v), was then added and samples were vortexed simultaneously for 1 min. After centrifugation at 3000 g at 2°C for 10 min, 400 μl of the upper phase containing 32P was removed for counting in 5 ml scintillant. Ca-activated ATPase activity is defined as the difference in P liberation measured in the presence of calcium and 4 mM EGTA. Ca-ATPase activity was linear with protein concentration up to 0.2 mg/ml (twice that used in the assay). Basal and Ca-activated ATP hydrolysis was linear with time. Samples were assayed and Ca-ATPase activity computed in duplicate. The linear regression analysis of the line determined by plotting
Table 1. Regional distribution of individual lens ATPase activity

<table>
<thead>
<tr>
<th>Individual rabbit lens</th>
<th>Ca-ATPase activity (nmol P/mg protein/hr)</th>
<th>Na-K-ATPase activity (nmol P/mg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelium†</td>
<td>140 ± 29</td>
<td>725 ± 18</td>
</tr>
<tr>
<td>Cortex</td>
<td>78 ± 12</td>
<td>359 ± 18</td>
</tr>
<tr>
<td>Nucleus</td>
<td>n.d.</td>
<td>289 ± 68</td>
</tr>
</tbody>
</table>

*Maximal Ca-ATPase activity measured at 10^-5 M[Ca^{2+}]. ATPase activity ± standard error, n = 3 for epithelium, n = 8 for cortex and nucleus.
† Pooled epithelium from two lenses from same animals, n = 3 pools.

Results

The experimental protocol for the preparation of lens material for ATPase determination was developed with the aim of obtaining a sample enriched with cell membranes. Using this methodology, the lipid:protein ratio was increased 22- and 33-fold in the bovine lens cortical and nuclear membrane preparations, respectively. The total phospholipid recovery was 94%, indicating little loss of membrane during the preparation procedure.

Pooled lens tissue was used to obtain sufficient material for careful characterization. Individual rabbit lens studies were also conducted and the data are presented in Table 1. Unless indicated, presentation and discussion of results refer to the pooled lens study.

Examination of the enriched lens membrane preparation by electron microscopy (Fig. 1) revealed that ATP hydrolysis vs. time (six data points) gives an average correlation coefficient 0.991 ± 0.016.

Na-K-ATPase activity was measured at 37°C in 350 μl of a reaction mixture containing 40 mM histidine-HCl, pH 7.4, 10 mM KCl, 3 mM MgCl₂, 0.8 mM EGTA (buffered with tris pH 7.4), 100 mM NaCl, 5 mM [γ-³²P]ATP (10⁶ cpm/μmol), microsomes (0.1 mg/ml protein), and either water or 1 mM ouabain. Stock 10 mM ouabain solution was prepared fresh for each experiment. ATP hydrolysis was terminated and ³²P liberation was determined for Ca-ATPase activity above. Ouabain sensitive Na-K-ATPase activity is defined as the difference in P liberation measured in the presence and absence of ouabain.

Only 0.3% of the total radioactivity in the absence of microsomes could be extracted from [γ-³²P]ATP by the phosphomolybdate isobutyl alcohol:benzene system, indicating negligible ATP hydrolysis of our stock ATP.

Fig. 1. Electron micrograph of bovine lens cortical fiber cell membranes prepared as described in the text. Original magnification ×23,200; bar = 1.0 μm.
in the preparation used for ATPase determinations, the lens membranes had formed vesicles. The presence of membranes in the form of vesicles can introduce artifacts into ATPase determinations by limiting the accessibility of ions to the inner-facing surface of the membrane. For this reason, lens membrane preparations were made permeable by including calcium ionophore in the buffers used in the Ca-ATPase assays.

In both the rabbit and bovine lens, Ca-ATPase activity was detected in the capsule-epithelium and in the cortex. Calcium ATPase activity is appreciable in the cortical membrane preparations and slightly higher in the epithelial membrane preparations whether the data are expressed per mg protein (Table 1), or when the Ca-ATPase activity is expressed in terms of the amount of phospholipid (Table 2), an indication of membrane surface area. Ca-ATPase activity was not detected in preparations of the lens nucleus of either species.

The pattern of stimulation of lens ATPase activity by varying concentrations of calcium was observed to be similar in both the rabbit and bovine lens (Fig. 2). At low calcium concentrations, increasing the calcium level increases the ATPase activity. However, maximal calcium-stimulated ATPase activity was observed at a calcium concentration of 10^{-4} M; further increasing the calcium concentration reduced ATPase activity. The concentration of calcium necessary for half-maximal stimulation of ATPase activity was approximately 10^{-6} M for both cortex and epithelium of each species. Basal Mg-ATPase activity was about 68% of maximal ATPase activity measured in the presence of calcium. The large standard deviations presented in Figure 2 mask the linearity of the calcium stimulation curve. The large deviations arise from three to four experiments which are very linear in themselves, but are displaced up and down along the Y axis. From the linear regression analysis of the log [Ca] (M) vs. ATP hydrolysis (nmoles/mg/hr) for each experiment, a P value much less than 0.01 is calculated for each experiment except for the four bovine epithelium experiments which resulted in an average P value of 0.03 ± 0.029.

Na-K-ATPase activity was also measured. The regional distribution of Na-K-ATPase activity measured within both the rabbit and bovine lens (Table 1) is similar to that observed by other investigators^{30-32}; the activity in the nucleus and cortex is less than in the epithelium. In the lens epithelium and cortex, Na-K-ATPase activity was observed to be five times greater than Ca-ATPase activity (Table 1).

Ca-ATPase from individual lenses could be measured with a precision of about ±30%. Adequate cortical membrane tissue can be obtained for 15-20 Ca-ATPase determinations. Much of the absolute error associated with measuring Ca-ATPase activity in the individual epithelial preparations is in measuring the protein concentration. At best, two Ca-ATPase determinations may be made using epithelium from a single lens.

**Discussion**

The maintenance of a low intracellular free calcium concentration appears to be vital to the well-being of the lens, as it is in most other tissues. The association of elevated lenticular calcium levels, with lens opacification, has been recognized for many decades^{8} and specific links between calcium and lens transparency have recently been suggested.^{7,10}

Since the gradient of free calcium concentration between the lens cytoplasm and the external medium is so great, it might be expected that some calcium...
Rabbit Ca-ATPase Activity

Fig. 2. Calcium dependency of Ca-ATPase activity of membrane enriched rabbit (A), and bovine (B) lens preparations. Activity is expressed as nmoles phosphate released per mg protein per hr at 37°C, pH 7.4. Calcium was buffered with EGTA, Kd = 1 × 10^-6.

- - - Cortex, - - - Epithelium.

continually leaks passively into the intracellular compartment of the lens. To balance the inward leak, the lens must be able to extrude that calcium. Studies of Ca-ATPase activity have been undertaken to elucidate the active calcium pump mechanism. Calcium-ATPase activity has been determined in the lens epithelium but only a very low activity of Ca-ATPase within the lens mass fiber has been reported. However, the lens fiber mass has an unusually high protein content which could interfere with the measurement of Ca-ATPase. For this reason, we set out to determine whether Ca-ATPase in the lens fiber mass could be more reliably detected using a membrane-enriched preparation of the tissue which frees the membrane from much of the potential interference due to the cytoplasmic proteins.

Using membrane-enriched preparations of both the bovine and rabbit lens, substantial Ca-ATPase activity was determined in both the lens cortex and the lens epithelium. In fact, the specific activity of the Ca-ATPase in the lens epithelium was only slightly higher than that in the lens cortex. Given the relative mass of the lens cortex compared to the epithelium, this means that the bulk proportion of the total potential Ca-ATPase activity in the lens is distributed within the cortex. No Ca-ATPase activity was detected in the nucleus. It is possible that Ca-ATPase activity is suppressed in the nuclear region because of the high membrane cholesterol content and possible increased order of the membranes in the lens nucleus membrane. Lipid order and cholesterol have been shown to diminish Ca-ATPase activity in other tissues and model membranes.

Membrane lipid order has much less influence upon Na-K-ATPase activity. Preliminary attempts to activate the Ca-ATPase activity on our nuclear and cortical preparations with exogenous calmodulin or protein kinase catalytic subunit have been negative.

The calcium activation curves for the ATPase in membrane-enriched lens cortex and epithelial samples show low concentrations of calcium-stimulated ATPase activity, while high calcium levels inhibit the enzyme further. It is possible that calcium is inhibiting basal ATPase activity, however, in other tissues where basal activity is absent, it has been suggested that at high calcium concentrations the low affinity site of the pump binds calcium and transports it out of the cell, hydrolysing ATP; above 10^-3 M calcium, the low affinity site of the pump binds calcium, the pump is reversed, and ATP is synthesised from ADP and inorganic phosphate.

The calcium concentration required to elicit half-maximal Ca-ATPase activity in the lens fractions ranged from 0.6 × 10^-6 to 2 × 10^-6 M, which is in the range of the values measured for the Ca-ATPase in cardiac sarcoplasmic reticulum (0.87-1.05 × 10^-6 M). The present observations have indicated that the specific activity of Ca-ATPase in lens cortical fibers is similar to that of the epithelium of both the bovine and rabbit lens. The presence of such a substantial quantity of calcium transport enzyme in the lens cortex might be linked to the lack of organelles in the lens fibers. In other cell types, inward leakage of calcium is dealt with both by extrusion back into the
extracellular space and by sequestration in intracellular organelles. Lacking the intracellular organelles generally responsible for sequestering calcium, lens fibers presumably must rely exclusively on active calcium extrusion.

Key words: calcium-ATPase, lens, membrane, cortex, epithelium, bovine, rabbit

References