A new member of the ATPase family

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Czerwinski, A., H. J. Gitelman, and L. G. Welt. A new member of the ATPase family. Am. J. Physiol. 213(3): 786-792. 1967.—Rat erythrocyte ghosts have an ATPase activity which is magnesium dependent, sodium activated, but potassium inhibited. The sodium activation is completely inhibited by cardiac glycosides. The glycoside inhibition is, in turn, blocked by potassium. Calcium also inhibits the sodium activation of this ATPase. This ATPase is seen only at low concentrations of ATP substrate and has a \( K_m \) of 2.2 \( \times 10^{-7} \) \( \text{m} \) ATP/liter. The possibilities that it may be involved in the facilitated transport of hexoses and of amino acids are suggested as is the possibility that it may be involved in a noncoupled sodium transport.

Rat; erythrocyte ghosts

**METHODS**

The preparation of rat erythrocytic membranes involves three consecutive steps: hemolysis of the erythrocytes, washing the hemolysate with a sodium-containing solution, and a final wash with a sodium-free solution. The hemolyzing solution contained 1 \( \times 10^{-4} \) \( \text{m} \) disodium ethylenediaminetetraacetic acid (EDTA) and sufficient 1 \( \times 10^{-5} \) \( \text{m} \) tris (hydroxymethyl) amino methanc to stabilize the \( \text{pH} \) of the solution between 7.4 and 7.6. The sodium wash solution contained 0.015 \( \text{m} \) NaCl, 0.0017 \( \text{m} \) tris, and 1 \( \times 10^{-4} \) \( \text{m} \) disodium EDTA with additional 1 \( \times 10^{-5} \) \( \text{m} \) tris added if the \( \text{pH} \) was below 7.4. The sodium-free wash solution contained 1 \( \times 10^{-4} \) \( \text{m} \) of the acid salt of EDTA and sufficient 1 \( \times 10^{-5} \) \( \text{m} \) tris to bring the \( \text{pH} \) to between 7.4 and 7.6. Hemolysis and centrifugation were carried out at 10-20 C and a Sorvall RC-2 refrigerated centrifuge was used throughout preparation. This temperature was employed because it was found that the exposure of rat erythrocytes to a temperature less than 10 C makes it impossible to rid the hemolyzed cells of their hemoglobin.

Sprague-Dawley rats weighing 150-250 g were anesthetized with Nembutal and exsanguinated by aortic puncture. The blood was mixed with ammonium heparin and centrifuged so that the plasma could be removed. The remaining lightly packed erythrocytes were added to 10 vol of vigorously agitated hemolyzing solution, and after 20 min the hemolysate was centrifuged at 30,000 \( X \)g for 30 min. The supernate was discarded and the remaining red pellet of membranes was resuspended in 0.15 \( \text{m} \) NaCl. This suspension was recentrifuged at 30,000 \( X \)g for 30 min, the buffy coat removed, the supernate discarded, and the membrane pellet rehemolyzed as previously described.

Following the second hemolysis and centrifugation, the membranes were suspended in 10-20 vol of sodium-containing wash solution and then centrifuged at 30,000 \( X \)g for 20 min. Again, the supernate was discarded, fresh wash solution added, and the membranes recentrifuged. This step was repeated until the supernate was colorless (usually 3-6 washes). If at any time during washing a distinct two-layered pellet was seen, the lighter membranes were decanted and the button with more hemoglobin was discarded.

At this point the membranes were usually pink in color and the cells were suspended in the sodium-free wash solution and centrifuged as described previously to remove as much hemoglobin, sodium, and potassium as possible. This step was repeated until the supernate was again colorless, or for a minimum of three times. Follow-
A NEW MEMBER OF THE ATPase FAMILY

FIG. 1. Effect of magnesium and magnesium plus sodium on ATPase activity.

In those studies in which intact erythrocytes were used, the heparinized blood repeatedly was centrifuged and washed with isotonic saline to remove plasma and buffy coat. One milliliter of diluted cells was then added to 9.0 ml of the appropriate salt and ATP solution so that the final ATP concentration was $2 \times 10^{-6}$ M. This preparation was then incubated at 37°C for 30 min and thereafter the cells and supernate separated by centrifuging at 30,000 Xg for 10 min. The supernate thus obtained was divided and one-half was reincubated an additional 30 min. The purpose of this second incubation was to determine whether, in the prior steps, ATPase had become dissociated from the intact erythrocytes. Except for this additional incubation, both halves of the supernate, after incubation, had enzymatic ATPase activity terminated by plunging the tubes in boiling water for 2 min and the amount of ATP that had disappeared was estimated as previously described.

The phosphorylated nucleotide products of the enzyme reaction were examined by paper chromatography (21) and inorganic phosphate was measured by an automated Lowry-Lopez technique (11). The quantity of ADP produced was estimated by taking 2.0 ml of supernate from tubes in which enzymatic hydrolysis of ATP had occurred and adding 0.25 ml 0.17 M KCl, 0.25 ml $4 \times 10^{-5}$ M phospho enol pyruvic acid (Sigma Chemical Co.), and 0.01 ml pyruvate kinase (C. F. Boehringer & Soehne, Germany). These tubes, along with tubes containing a measured amount of ADP, to test the adequacy of ATP regeneration, were then incubated at 37°C for 15 min and thereafter assayed for ATP by the luciferin-luciferase method. From the amount of ATP present prior to hydrolysis, the amount of ATP remaining after enzymatic hydrolysis, and the amount of ATP regenerated after incubation with phospho enol pyruvic acid and pyruvate kinase, the quantity of ADP produced during exposure to ATPase was calculated.

In the remainder of the paper the following operational definitions are used: Total ATPase activity represents...
the amount of ATP hydrolyzed after incubation with erythrocytic membranes or intact cells and is quantitated in pm (10^-12 M) ATP hydrolyzed per minute per 1.0 mg of dried membranes or 1.0 ml of packed erythrocytes. Magnesium, or magnesium calcium ATPase, represents the amount of ATP hydrolyzed when erythrocytic membranes were incubated in a salt solution containing only magnesium or magnesium and calcium. Finally, sodium ATPase represents the total ATP hydrolyzed by membranes in a magnesium and sodium, or a magnesium, calcium, and sodium salt solution minus the ATP hydrolyzed in a magnesium or magnesium and calcium salt solution.

RESULTS

Cationic requirements. During the early phases of study the cationic requirements of this new ATPase were determined by adding magnesium, calcium, sodium, and potassium singly and then in varied mixtures to determine their effect on ATPase activity. In the absence of added cations ATPase activity was not demonstrable; however, when magnesium was added (Fig. 1), the ATPase activity was maximum at a magnesium concentration of 1.0 mM. Furthermore, when 100 mM sodium were added, there was an increase in total ATPase activity at each magnesium concentration, and the sodium-activated increment was maximum and approximately the same over a magnesium concentration of 0.5–2.0 mM.

The sodium activated increment was then studied, keeping the magnesium concentration constant at 1.0 mM and varying the sodium concentration from 0.06 to 200 mM (Fig. 2). The plot of sodium-activated ATPase vs. sodium concentration assumed a hyperbolic curve. Maximum ATPase activity obtained at a sodium concentration of 100 mM and plots of the reciprocals of sodium concentration and sodium ATPase activity revealed a mean one-half maximum concentration for activation by sodium of 13.9 mM.

When a similar study was performed, but with potassium in lieu of sodium, the total ATPase activity in the presence of magnesium and potassium was the same as the ATPase activity in the presence of magnesium alone. However, when magnesium, sodium, and varying concentrations of potassium were incubated with erythrocytic membranes, the sodium activated increment was partially inhibited (Fig. 3), and the concentration of potassium required to cause inhibition increased with increasing concentrations of sodium. It was determined that potassium acts as a competitive inhibitor of sodium by plotting the potassium concentration vs. the reciprocal of the sodium-activated ATPase at two sodium concentrations (Fig. 4). No study demonstrated more than 65% inhibition of the sodium-activated ATPase at an ATP concentration of 2 × 10^-6 M, and after attaining maximum inhibition further increases of potassium to levels as high as 25 mM caused only a gradually increasing inhibitory effect. Concentration for maximum and one-half maximum inhibition and the inhibitory constant is indicated in Table 1.

A further characterization of potassium inhibition was determined in the presence of 1.0 mM magnesium, 100 mM sodium, two ATP concentrations, and varying potassium concentrations. These results are plotted as shown in Fig. 5, and indicate that potassium acts also as a competitive inhibitor of ATP. The representative potassium concentrations for maximum and one-half maximum inhibition and the inhibitor constant are as indicated in Table 2. One major difference between these studies and those made at two sodium concentrations was that the percent inhibition of the sodium-activated ATPase was 65% at an ATP concentration of 1.0 × 10^-6 M and only 27% at an ATP concentration of 4.0 × 10^-6 M. This difference may indicate that a heterogeneous enzyme system is under study, or that potassium only weakly inhibits a sodium ATP complex and that increasing the
ATP concentration may overcome this inhibition by a mass action relationship.

The effect of calcium was more difficult to ascertain for not only was a magnesium ATPase demonstrated but also the presence of a calcium ATPase. Hence, it was first necessary to show that sodium had no effect on the calcium ATPase, and then the effect of calcium on the magnesium-dependent, sodium-activated ATPase was sought by maintaining the sodium concentration at 100 mM and varying the divalent cation concentration. To exclude an effect due to increasing the concentration of divalent cations per se in one set of tubes, the magnesium concentration was varied from 1.0 to 7.0 mM and, in a second set, the magnesium was maintained at 1.0 mM and the calcium varied from 0.25 to 6.0 mM. Thus, as is indicated in Fig. 6, an increase in the total divalent cation resulted in a small decrease in sodium ATPase activity, whereas the substitution of magnesium by increasing concentrations of calcium resulted in nearly complete inhibition of the sodium-activated ATPase.

Michaelis constant. To determine the effect of substrate concentration on the velocity of the reaction catalyzed by the sodium activated ATPase, the ATP concentration was varied from $1.0 \times 10^{-7}$ to $2.0 \times 10^{-6}$ M, whereas the magnesium and sodium concentrations were maintained at 1.0 and 100 mM, respectively. Results from such titrations demonstrated that the maximum velocity was approached when the ATP concentration exceeded $8 \times 10^{-7}$ M and, by plotting the reciprocals of the sodium-activated ATPase and ATP concentration, the Michaelis constant ($K_M$) was determined. As seen in Fig. 7, this is equivalent to $2.2 \times 10^{-7}$ M ATP.

Products of the reaction. The next problem was concerned with the products of the hydrolysis of ATP. Did the enzyme catalyze the hydrolysis of ATP to ADP and inorganic phosphate, to AMP and inorganic phosphate, or was there evidence of some other phosphorylated intermediate? To answer these questions, the end products were studied by paper chromatography and only one nucleotide was demonstrated, namely ADP. To further demonstrate that ADP was the major nucleotide produced, an ATP regeneration study was performed as described in Methods (Fig. 8). Bar 1 indicates the quantity of ATP initially added, bar 2 the amount remaining at the termination of the ATPase reaction, and bar 3 indicates the amount of ATP present after regenerating ADP to ATP. In each instance the quantity of ATP present after regeneration was virtually the same as that added initially.

The other major product, inorganic phosphate, was evaluated as follows: ATP disappearance and the appearance of inorganic phosphate were measured and a calculation made assuming that each mole of ATP that disappeared yielded 1 mole inorganic phosphate. As indicated in Table 3, the calculated and measured amounts of inorganic phosphate show good agreement and are compatible with the hypothesis that each mole of ATP which was hydrolyzed yielded 1 mole inorganic phosphate. Therefore, of the possibilities alluded to earlier, the most probable is that this enzyme catalyzed the hydrolysis of ATP to ADP and inorganic phosphate.

Effect of drugs. Since this ATPase may be concerned with active transport, the effect of certain known inhibitors of active transport (10, 15) was studied using corticosterone and the cardiac glycosides ouabain and scillaren A.

The effect of ouabain, a water-soluble glycoside, was determined by comparing the membrane ATPase activity in the absence and presence of drug. When present the drug concentration varied from $1 \times 10^{-8}$ to $1 \times 10^{-3}$ M; but, because of limited solubility, concentrations in excess of $10^{-3}$ M could not be used. In these concentrations ouabain had no effect on the magnesium ATPase; however, the sodium-activated increment was inhibited.

### Table 1. Summary effect of sodium on potassium inhibition

<table>
<thead>
<tr>
<th>Sodium</th>
<th>Potassium, mM</th>
<th>Maximal inhibition</th>
<th>One-half maximal inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 mM</td>
<td>3.5</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>100 mM</td>
<td>15.0</td>
<td>6.3</td>
<td></td>
</tr>
</tbody>
</table>

| Inhibitor constant | 0.24 |

### Table 2. Summary effect of ATP on potassium inhibition

<table>
<thead>
<tr>
<th>ATP</th>
<th>Potassium, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1 \times 10^{-5}$ M</td>
<td>10.0</td>
</tr>
<tr>
<td>$4 \times 10^{-5}$ M</td>
<td>15.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Maximal inhibition</th>
<th>One-half maximal inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.8</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Inhibitor constant | 1.5

FIG. 5. $K_i$ for potassium and ATP.
Effect of magnesium and calcium on sodium-activated ATPase

(Fig. 9), with one-half maximum inhibition at a ouabain concentration of $2.2 \times 10^{-6}$ M and complete inhibition at a concentration of $1 \times 10^{-5}$ M. The studies were then repeated in the presence of added potassium, 15 mM, and under these circumstances the ouabain inhibition was no longer discernible. Therefore, in the absence of potassium ouabain can completely inhibit the sodium-activated ATPase, but in the presence of potassium this inhibition was blocked.

Similar experiments were carried out with an alcohol-soluble glycoside, scillaren A. However, a problem not studied further concerned an alteration in the sodium ATPase when alcohol alone was added to the incubation media. Because of this, exact data concerning the concentration of scillaren A for one-half maximum inhibition of the sodium ATPase were not obtained, but apparently maximum and complete inhibition was obtained at a scillaren concentration of $1 \times 10^{-4}$ M.

Corticosterone in concentrations varying from $1 \times 10^{-8}$ to $1 \times 10^{-8}$ M was tested with erythrocytic membranes from intact and adrenalectomized rats. In each instance this drug had no demonstrable effect on either the magnesium or sodium ATPase's.

Localization of the sodium ATPase. Having determined the operational characteristics of the ATPase, our next concern was where an enzyme, requiring magnesium and high-sodium concentrations, and inhibited by potassium, calcium, and cardiac glycoside, could be localized in the erythrocytic membrane. The cationic specifications make it unlikely that this enzyme is located on the inner surface. Two other possibilities must be considered: 1) that the enzyme is located on the outer membrane surface or 2) that it is within the membrane. The first hypothesis was tested by incubating intact erythrocytes in isosmotic solutions containing ATP and magnesium and sodium; magnesium, sodium, and ouabain; and magnesium, sodium, and calcium. Under these circumstances, although ATP disappeared, the rate of disappearance was not altered by the concentrations of potassium, calcium, and cardiac glycosides which were of known effectiveness in the membrane preparation. This minor hydrolysis presumably represents a nonenzymatic reaction. Thus, of the possibilities considered, it seems most probable that this ATPase is compartmentalized within the cell membrane.

DISCUSSION

In evaluating a study such as this, one must recognize that an operational definition of an enzyme-like effect is under consideration. This limitation arises in major part because "membrane ATPase" has not been isolated and purified to allow characterization of the enzyme or enzymes. Thus, whether this represents a new ATPase or merely another facet of a previously described ATPase can only be presumed.

One major difference between this study and many of the previous studies concerned with membrane ATPase is that the ATP concentration was lower, $2 \times 10^{-6}$ M compared with $10^{-5}$-10^{-4}$ M ATP. This may also explain why a sodium-activated, potassium-inhibited ATPase has not been previously described, for at higher concentrations of ATP the percent contribution by this new enzyme system becomes trivial and is easily masked. Accordingly, it is noted that the new ATPase contributes up to 50% of the total ATPase activity at an ATP concentration of $2 \times 10^{-6}$ M, but because of the increase in total ATPase activity this enzyme can only contribute 5% of the total activity at an ATP concentration of $1 \times 10^{-4}$ M.

There are certain similarities and significant dissimilarities between this sodium activated ATPase and the alkali metal-sensitive ATPase described by Skou (17, 18), by Post et al. (14), and by Dunham (4). Both enzymes hydrolyze ATP and both require magnesium to demonstrate ATPase activity. However, contrary to the study of Skou (17, 18), a fixed optimum ratio of magnesium to ATP was not observed for this new ATPase. Furthermore, although this ATPase is maximally activated by the addition of sodium alone, the
A NEW MEMBER OF THE ATPase FAMILY

FIG. 8. Loss and recovery of ATP.

TABLE 3. Inorganic phosphate released from ATP by ATPase

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Inorganic Phosphate Released (× 10⁻⁵ M)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Calculated</td>
</tr>
<tr>
<td>1</td>
<td>Mg alone</td>
</tr>
<tr>
<td></td>
<td>Mg + Na</td>
</tr>
<tr>
<td>2</td>
<td>Mg alone</td>
</tr>
<tr>
<td></td>
<td>Mg + Na</td>
</tr>
<tr>
<td>3</td>
<td>Mg alone</td>
</tr>
<tr>
<td></td>
<td>Mg + Na</td>
</tr>
</tbody>
</table>

other alkali metal-sensitive ATPase is maximally activated only when sodium and potassium are present simultaneously (14, 17, 18). Indeed, the most significant difference between these two enzyme systems concerns the effect of potassium, for, whereas the enzyme of

REFERENCES

7. Järfelt, J. Properties and possible mechanisms of the Na⁺


