Increase in Adenosine Triphosphatase Activity of Ehrlich Ascites Tumor Cells Following Serial Cultivation in Media with Increased (Hypertonic) NaCl Content

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The cellular activity of ouabain-sensitive sodium-potassium activated adenosine triphosphatase (Na⁺⁺-K⁺⁺-ATPase) and ouabain-insensitive Mg⁺⁺-ATPase was analysed in sublines of Ehrlich ascites tumor cells serially cultivated either in isotonic media or in media with progressively increased NaCl concentration (up to 0.40 M).

Progressive elevation in the NaCl content of the medium resulted in a progressive increase of cellular ATPase activity, Na⁺⁺-K⁺⁺-ATPase thereby being more affected than Mg⁺⁺-ATPase. These alterations in ATPase activities are interpreted as "adaptive" changes in response to the elevated ionic environment, since "readaptation" of these "high-salt"-tolerant cells to growth in isotonic media resulted in a reversion of the enzyme activities to the level characteristic of the original parent cells.

Materials and Methods

The maintenance of these predominantly hyper-diploid Ehrlich ascites tumor cells in culture and the establishment of sublines in media with increased NaCl content ("high-salt"-tolerant cells) have been described elsewhere. The "high-salt"-tolerant cultures were subcultivated at least 10 times in their respective "high-salt" media before being used in the present experiments.

Monolayer cultures for the present experiments were incubated at 37 °C in 15 ml volumes of medium in 4-ounce pharmacy bottles (Fa. Federated Distributors) which contained an atmosphere of 20% O₂, 73% N₂ and 7% CO₂. The medium was replaced with 15 ml of fresh prewarmed (37 °C) medium every 24—48 hours and the incubation continued until the total cell number amounted to approximately 5—10 x 10⁶ cells per culture. The last medium renewal was performed always one hour before the cultures were utilized for enzyme determination. Cells were harvested by mechanical dislodging ("rubber policeman") from the glass surface. An aliquot of the cell suspension was used for replicate cell counts in a hemocytometer. Another aliquot containing ca. 5 x 10⁶ cells was centrifuged for 2 min at ca. 1000 g. The sediment was washed once with 10 ml of Earle's salt solution and once with 10 ml of 0.15 M Tris·HCl (pH 7.2). Then, the pellet was resuspended in 1 ml of 0.15 M Tris·HCl (pH 7.2) and the cells were broken by sonication for 10 sec at 0 °C, employing a Branson LS75 sonifier equipped with a micro-probe at an energy setting of ca. 50% maximum power. This treatment totally fragmented the cells as judged by microscopy examination. The sonicate was immediately assayed for ATPase activity.

Sublines of monolayer cultures of Ehrlich ascites tumor cells have been adapted, by gradually increasing the NaCl content of the culture media, to proliferation in hypertonic media at salt concentrations of 0.25, 0.30, 0.35, 0.40, 0.45 and 0.50 M, respectively. These cultures provide an in-vitro system of homogeneous mammalian cell populations for the study of long-term influences of a "high-salt" environment on cellular metabolism, and for the analysis of mechanisms which enable these cells to tolerate such high extracellular salt concentrations.

Since it is conceivable that these cells have to extrude sodium at an increased rate in order to prevent drastic intracellular ionic alterations in the presence of elevated external salinity, possible effects of this "high-salt" environment on the cellular ionic transport system are of particular interest. The membrane-bound (sodium plus potassium)-activated adenosine triphosphatase (Na⁺⁺−K⁺⁺−ATPase; EC 3.6.1.3.) is assumed to have an essential function in the active transport of Na⁺ and K⁺ across cellular membranes. The present investigation was therefore designed to investigate whether the Na⁺⁺−K⁺⁺−ATPase activity of Ehrlich ascites tumor cells in culture had changed after adaptation of these cells to proliferation in media with increased (hypertonic) NaCl content.

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**ATPase activity** was assayed by determination of the liberated inorganic phosphate in a medium (1 ml; pH 7.2; 25 °C) containing 100 mM Tris-HCl, 10 mM ATP, 10 mM MgCl₂, 50 mM NaCl, 20 mM KCl and ca. 0.5 - 1 x 10⁻⁶ sonicated cells. Under the conditions employed, ATP hydrolysis was a linear function of time (at least for 90 min) and of enzyme concentration. The Kₘ for ATP was observed to be approximately 2 mM. The activity of Na⁺-K⁺-ATPase was assumed to be the fraction which was sensitive to inhibition by ouabain (1 mM). This activity corresponded roughly to the difference between the value obtained in the presence of Na⁺, K⁺ plus Mg²⁺ and the value obtained in the presence of Mg²⁺ alone. The amount of ATP breakdown which was insensitive to inhibition by ouabain (1 mM) is referred to as Mg²⁺-ATPase. ATPase activity is expressed as micromoles of inorganic phosphate liberated per 10⁷ cells per min at 25 °C.

**Designation of cultures:** ED-0.15 ("control"), ED-0.25, ED-0.30, ED-0.35 and ED-0.40 cultures refer to sublines of Ehrlich ascites tumor cells growing in media with different NaCl content (Moles/l): 0.15, 0.25, 0.30, 0.35 and 0.40. ED-N-0.15 designates cultures which have been adapted to proliferation in 0.15-M calf serum (salt concentration: 0.15 M). ED-0.25- up to 0.40: exponentially growing cultures in media containing different concentrations of NaCl. ED-0.15- and ED-N-0.15-cells). The present results obtained with "defined", homogeneous populations of mammalian cells in culture extend and confirm observations of several authors who have demonstrated changes of Na⁺-K⁺-ATPase activity in response to osmotic stresses in a variety of tissues of different species, as in the salt glands of marine birds, in the intestinal mucosa, kidney and gills of fish, and the outer kidney medulla of rabbits. The demonstration (Table 1) of an enhanced activity of Mg²⁺-ATPase in "high-salt"-tolerant cells is in accordance with findings about the effect of changes of the salt load in the drinking water on the Mg²⁺-ATPase activity of the salt glands of ducks and gulls. On the other hand, no effect of increased environmental osmolality on Mg²⁺-ATPase activity of gills, intestine or kidney of freshwater fish was reported when these fish were adapted to seawater. The different findings in regard to Mg²⁺-ATPase may be accounted for by differences in the species and/or techniques used in these experiments.

It may be inferred from the present results that the increased NaCl concentration in the environment of these "high-salt"-tolerant cells resulted in the adaptive rise of cellular ATPase activity, Na⁺-K⁺-ATPase thereby being more affected than Mg²⁺-ATPase. The findings strongly support the concept that this (these) enzyme(s) are intimately associated with the active cation transport across cellular membranes. Thus, the predominantly enhanced activity of the presumable "sodium-pump" might be interpreted as an expression of an increased rate of Na⁺-K⁺-ATPase in response to the elevated ionic environment appeared to be more striking than the increase of Mg²⁺-ATPase activity (Table 1). Thus, the increase of specific activity of Na⁺-K⁺-ATPase in e.g. ED-0.40-cells (in comparison with ED-0.15-cells) was found to be about tenfold, while Mg²⁺-ATPase had increased only by a factor of four. The gradual elevation of cellular ATPase activity concomitant with increasing extra-cellular NaCl concentration might be regarded as an adaptive change in response to the environmental ionic changes. In favour of such an adaptive mechanism is also the finding that "readaptation" of "high-salt"-tolerant cells (e.g. ED-0.40-cells) to growth in isotonic media resulted in a nearly complete reversion in ATPase activity to the level characteristic of the original parent cells (Table 1: compare activities of ED-0.15- and ED-N-0.15-cells). The different observations of several authors who have demonstrated changes of Na⁺-K⁺-ATPase activity in response to osmotic stresses in a variety of tissues of different species, as in the salt glands of marine birds, in the intestinal mucosa, kidney and gills of fish, and the outer kidney medulla of rabbits. The demonstration (Table 1) of an enhanced activity of Mg²⁺-ATPase in "high-salt"-tolerant cells is in accordance with findings about the effect of changes of the salt load in the drinking water on the Mg²⁺-ATPase activity of the salt glands of ducks and gulls. On the other hand, no effect of increased environmental osmolality on Mg²⁺-ATPase activity of gills, intestine or kidney of freshwater fish was reported when these fish were adapted to seawater. The different findings in regard to Mg²⁺-ATPase may be accounted for by differences in the species and/or techniques used in these experiments.

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<table>
<thead>
<tr>
<th>Enzyme activity (μmoles Pi/10⁷ cells/min; means ± S.D.)</th>
<th>Na⁺-K⁺-ATPase</th>
<th>Mg²⁺-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>cells *</td>
<td>n</td>
<td></td>
</tr>
<tr>
<td>ED-0.15</td>
<td>20</td>
<td>1.03 ± 0.3 (100)**</td>
</tr>
<tr>
<td>-0.25</td>
<td>15</td>
<td>4.15 ± 1.0 (403)</td>
</tr>
<tr>
<td>-0.30</td>
<td>15</td>
<td>5.78 ± 1.2 (561)</td>
</tr>
<tr>
<td>-0.35</td>
<td>11</td>
<td>8.70 ± 1.7 (845)</td>
</tr>
<tr>
<td>-0.40</td>
<td>6</td>
<td>10.72 ± 0.9 (1041)</td>
</tr>
<tr>
<td>-0.15</td>
<td>6</td>
<td>1.75 ± 0.4 (170)</td>
</tr>
</tbody>
</table>

Table 1. Mean cellular activity of Na⁺-K⁺-ATPase and Mg²⁺-ATPase in Ehrlich ascites tumor cells from exponentially growing cultures in media containing different concentrations of NaCl.

n = number of cultures analyzed. ** = ED-0.15-cells ("control") in Eagle's basal medium supplemented with 10% fetal calf serum (salt concentration: 0.15 M). ED-0.25- up to 0.40: "high-salt"-tolerant cells in media with increased NaCl content (salt concentration: 0.25 up to 0.40 M). ED-N-0.15-cells "readapted" to growth in control medium after serial cultivation in media with increased NaCl content (0.40 M).
active sodium transport in order to prevent drastic intracellular ionic alterations in the presence of elevated external salinity. Since adenosine triphosphate (ATP) is the "fuel" for this active transport process, one would also expect an increase in the activity of metabolic pathways supplying ATP. The demonstration of an increased glucose catabolism and an elevated oxygen consumption of these "high-salt"-tolerant cells will be described elsewhere.\textsuperscript{1,27}

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