Changes in Microsomal Na\(^+\), K\(^+\), Mg\(^{2+}\) and Ca\(^{2+}\)-ATPase Activities during Proliferation of Chinese Hamster V-79 and Human HeLaS-3 Cells.

Yukiko Kunugi-Uehara, Junji Miyakoshi, Wasako Oda and Chiyoko Inagaki

Department of Biology, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607, Japan

ABSTRACT. Changes in microsomal Na\(^+\), K\(^+\), Mg\(^{2+}\) and Ca\(^{2+}\)-ATPase activities during cell proliferation were examined in Chinese hamster V-79 (V-79) cells (normal cells) and human HeLaS-3 (HeLaS-3) cells (malignant cells). For V-79 cells, the Mg\(^{2+}\)-ATPase activity per cell (pmol Pi/h/cell) in the confluent phase was higher than that in the logarithmically growing (log) phase. The amount of microsomal protein per cell was also high in the confluent phase. Specific activities (fmol Pi/h/mg protein) of Na\(^+\), K\(^+\), Mg\(^{2+}\) and Ca\(^{2+}\)-ATPases were significantly lower in the confluent phase than in the log phase. For HeLaS-3 cells, an increase in Ca\(^{2+}\)-ATPase activity per cell was observed. The amount of microsomal protein per cell did not change between the log and confluent phase. The specific activity of Ca\(^{2+}\)-ATPase in the confluent phase was also markedly higher than in the log phase. The relation between changes in ATPase activities and cell proliferation is discussed.

Contact inhibition of cell growth has been reported to be associated with changes in plasma membrane functions (1, 5, 12, 15). Transport activities for deoxyglucose (1, 5) and non-metabolizable amino acids (5, 12) in contact-inhibited cells are reduced with increases in cell density, but no changes occur in transformed cells that do not show contact inhibition. Ouabain-sensitive uptake of \(^{86}\)Rb\(^+\), as a K\(^+\) analogue, also decreases with increasing density of untransformed cells (10). The transport of sugars and amino acids is accelerated by the Na\(^+\) gradient across the plasma membrane (14), this Na\(^+\) gradient being formed by the Na\(^+\) extrusion and K\(^+\) uptake of cells with ouabain-sensitive and plasma membrane-located ATPase (Na\(^+\), K\(^+\)-ATPase) (3). Therefore, the ATPase activities of the plasma membrane are thought to change with cell density.

We here present an examination on changes in microsomal ATPase activities with relation to growth regulation based on results of the culture of nonmalignant and malignant cells.
MATERIALS AND METHODS

Cell cultures. Chinese hamster V-79 and HeLaS-3 cells were maintained in modified Eagle's minimum essential medium supplemented with 15% heat-inactivated bovine serum. Samples containing $5 \times 10^4$ cells were plated on Petri dishes ($100 \times 20$ mm) then grown under stationary conditions at 37°C in a humidified atmosphere of 85% air and 15% CO₂. The medium was changed 4 or 5 days after plating. Cells in the logarithmic growth (log) or confluent phase were collected 3 and 5 days after the plating of V-79 cells and 6 and 8 days after the plating of HeLaS-3 cells. Cells were collected by scraping the dishes with a rubber spatula then centrifuging the cell suspension at 1,500 g for 10 min. The cell concentration was measured with a Coulter counter.

Preparation of microsomal fractions. All procedures were done at 0-4°C. The collected cells were washed three times in cold saline then homogenized in a Teflon-glass homogenizer in cold buffer solution containing 0.25 M sucrose, 25 mM Tris-Mes (pH 7.4) and 1 mM EDTA. The homogenate was centrifuged at 10,000 × g for 10 min. The resulting supernatant was diluted 10-fold with 1 mM EDTA-Tris (pH 7.4) then centrifuged at 25,000 × g for 30 min. The pellet formed was resuspended in 5 mM EDTA-Tris (pH 7.4) and used as the microsomal fraction. The protein concentration was determined by the method of Lowry et al. (11).

Assay of ATPase activities. Unless otherwise stated, the reaction mixture contained 100 mM Tris-Mes (pH 7.4), 6 mM Mg(CH₃COO)₂, 100 mM NaCl, 10 mM KCl, 1 mM EDTA, 10-20 µg of microsomal protein and 6 mM ATP-Na₂ in a total volume of 200 µl. The enzyme reaction was started by adding ATP after a 5 min-equilibration of the reaction mixture at 37°C. Incubation was done at 37°C for 30 min then stopped by the addition of 200 µl of cold 10% trichloroacetic acid. After centrifugation of the reaction mixture at 1,500 × g for 10 min, 300 µl of its supernatant was pipetted out, and the inorganic phosphate liberated measured according to the method of Chen et al. (2).

Na⁺, K⁺-ATPase activity was calculated by subtracting the activity in the presence of 1 mM ouabain from the total ATPase activity. The activity measured in the presence of 1 mM ouabain was regarded as the Mg²⁺-ATPase activity. The Mg²⁺-ATPase activity in the presence of 0.3 mM EA was designated as the EA less sensitive Mg²⁺-ATPase activity. The difference between these activities in the presence and absence of 0.3 mM EA was designated as the EA highly sensitive Mg²⁺-ATPase activity. Ca²⁺-ATPase activity was assayed with 2 mM CaCl₂ in the reaction mixture described above. The difference between the activities in the presence and absence of 2 mM CaCl₂ was the Ca²⁺-ATPase activity.

Materials. Ouabain (Merck, Darmstadt, West Germany), ATP-Na₂ (Böhringer, München, West Germany) and EDTA (Nakarai Chemicals, Kyoto, Japan) were used. Ethacrynic acid was a gift from Merck, Sharp & Dohme Research Laboratories (West Point, PA, USA). All other chemicals used were of reagent grade.

Statistical analysis. The significance of the differences between values was analyzed by the paired t-test (4). Differences for p values below 0.05 were considered significant.

RESULTS

Microsomal ATPase activities were assayed for both V-79 and HeLaS-3 cells at the log and confluent phases of growth. The activities of Na⁺, K⁺ and Ca²⁺-ATPases per cell in the confluent phase of V-79 cells were similar to those in the log phase (Table 1). The Mg²⁺-ATPase activity per cell in the confluent phase was slightly higher...
ATPase Activities during Proliferation

TABLE 1. ACTIVITIES PER CELL OF MICROSOMAL ATPASES IN CHINESE HAMSTER V-79 AND HELAS-3 CELLS.

<table>
<thead>
<tr>
<th>Cell line/Phase</th>
<th>Activity per cell (pmol Pi/h/cell)</th>
<th>Na⁺,K⁺-ATPase</th>
<th>Mg²⁺-ATPase</th>
<th>Ca²⁺-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total EA highly sensitive EA less</td>
<td></td>
<td></td>
<td>sensitive</td>
</tr>
<tr>
<td></td>
<td>sensitive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chinese hamster</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-79</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>log</td>
<td>(6.1 ± 0.7) × 10⁶ 1.6 ± 0.2</td>
<td>0.65 ± 0.02</td>
<td>0.26 ± 0.02</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td>confluent</td>
<td>(1.4 ± 0.6) × 10⁷ 2.0 ± 0.2</td>
<td>0.80 ± 0.06</td>
<td>0.23 ± 0.02</td>
<td>0.55 ± 0.14</td>
</tr>
<tr>
<td>HeLaS-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>log</td>
<td>(7.0 ± 0.9) × 10⁶ 3.8 ± 0.2</td>
<td>1.4 ± 0.2</td>
<td>0.30 ± 0.05</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>confluent</td>
<td>(1.9 ± 0.6) × 10⁷ 3.9 ± 0.2</td>
<td>1.4 ± 0.2</td>
<td>0.30 ± 0.02</td>
<td>1.1 ± 0.2</td>
</tr>
</tbody>
</table>

Each value is the mean ± S.E. of 4–9 determinations. EA: ethacrynic acid.
* p<0.01 using the paired t-test (confluent vs. log).

TABLE 2. SPECIFIC ACTIVITIES OF MICROSOMAL ATPASES IN CHINESE HAMSTER V-79 AND HELAS-3 CELLS

<table>
<thead>
<tr>
<th>Cell line/Phase</th>
<th>Specific activity (μmol Pi/h/mg protein)</th>
<th>Na⁺,K⁺-ATPase</th>
<th>Mg²⁺-ATPase</th>
<th>Ca²⁺-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total EA highly sensitive EA less sensitive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chinese hamster</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V-79</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>log</td>
<td>0.24 ± 0.02</td>
<td>6.7 ± 0.6</td>
<td>2.7 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>confluent</td>
<td>0.38 ± 0.01*</td>
<td>5.2 ± 0.6*</td>
<td>2.1 ± 0.2*</td>
<td>0.6 ± 0.1*</td>
</tr>
<tr>
<td>HeLaS-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>log</td>
<td>0.69 ± 0.05</td>
<td>5.5 ± 0.5</td>
<td>2.1 ± 0.2</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>confluent</td>
<td>0.73 ± 0.06</td>
<td>5.3 ± 0.3</td>
<td>1.9 ± 0.3</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

Each value is the mean ± S.E. of 5–9 determinations. EA: ethacrynic acid.
* p<0.01 using the paired t-test (confluent vs. log).

than in the log phase. For the HeLaS-3 cells, no significant differences were detected between the log and confluent phases for Na⁺, K⁺- and Mg²⁺-ATPase activities per cell. The Ca²⁺-ATPase activity per cell in the confluent phase, however, was 5 times that in the log phase.

Mg²⁺-ATPase activity has been classified as being of two types according to its sensitivity to a potent diuretic, ethacrynic acid (7–9). We also measured these Mg²⁺-ATPase activities, which we designated EA highly sensitive and EA less sensitive Mg²⁺-ATPase activities (Table 1). The activities per cell of the EA highly sensitive Mg²⁺-ATPase in V-79 cells in the log and confluent phases were similar. The activity of the EA less sensitive Mg²⁺-ATPase per cell was higher in the confluent phase than in the log phase. For the HeLaS-3 cells, both the EA highly sensitive and EA less sensitive Mg²⁺-ATPases had similar activities per cell in the log and confluent phases.

The amount of microsomal protein per cell was measured, and specific activities of
the microsomal ATPases calculated based on the microsomal protein per cell obtained for V-79 and HeLaS-3 cells (Table 2). In V-79 cells, the amount of microsomal protein per cell was 1.5 times that in the log phase. Specific activities of the Na⁺, K⁺, Ca²⁺- and EA highly sensitive Mg²⁺-ATPases were significantly lower in the confluent phase, whereas the specific activity of the EA less sensitive Mg²⁺-ATPase

![Lineweaver-Burk plots of Na⁺,K⁺- and Mg²⁺-ATPases in Chinese hamster V-79 cells.](image)

Fig. 1. Lineweaver-Burk plots of Na⁺,K⁺- and Mg²⁺-ATPases in Chinese hamster V-79 cells. (A) Na⁺,K⁺-ATPase. (B) Mg²⁺-ATPase. (●—●) activity in the log phase. (○—○) activity in the confluent phase. Activities are specific (μmol Pi/h/mg protein).

![Sodium and potassium ion dependencies of Na⁺,K⁺-ATPase activities in different phases of Chinese hamster V-79 cells.](image)

Fig. 2. Sodium and potassium ion dependencies of Na⁺,K⁺-ATPase activities in different phases of Chinese hamster V-79 cells. (A) Na⁺ dependence. (B) K⁺ dependence. (●—●) activity in the log phase. (○—○) activity in the confluent phase. Activities are specific (μmol Pi/h/mg protein).
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The specific activities of the Na⁺, K⁺- and both types of Mg²⁺-ATPases in the HeLaS-3 cells in the confluent phase were similar to those in the log phase, but Ca²⁺-ATPase activity was higher in the confluent phase.

The kinetic parameters for the Na⁺, K⁺- and Mg²⁺-ATPases in V-79 cells were measured (Fig. 1). Lower Vₘₐₓ values were obtained in the confluent phase for both the Na⁺, K⁺- and Mg²⁺-ATPases, but the Kₘ values for ATP in the log and confluent phases were practically the same. Furthermore, the Na⁺, K⁺-ATPase in both phases showed nearly the same affinity for the cation cofactors, Na⁺ and K⁺ ions (Fig. 2). In the HeLaS-3 cells, both the Vₘₐₓ and Kₘ values for ATP in the confluent phase were similar to those in the log phase (data not shown).

In the study reported here, changes in microsomal ATPase activities followed characteristic patterns in the cell line used. V-79 cells used as a non-malignant cell line, showed an increase in total Mg²⁺-ATPase activity per cell, especially in the EA less sensitive Mg²⁺-ATPase activity per cell. The physiological role of microsomal Mg²⁺-ATPase, however, was not clear; but, the microsomal EA less sensitive Mg²⁺-ATPase has been found to be insensitive to a variety of monovalent ions including Na⁺, K⁺ and Cl⁻, whereas the EA highly sensitive Mg²⁺-ATPase activity is affected by Cl⁻ or NO₃⁻ (7-9). These findings suggest that an ATP-Mg-requiring, ion-insensitive system operates during the confluent phase of V-79 cells.

Our experiments revealed a marked increase in the microsomal protein concentration in the confluent phase of V-79 cells. The nature of these microsomal proteins, one of which probably has EA less sensitive Mg²⁺-ATPase activity, has still to be analyzed.

In the study reported here, changes in microsomal ATPase activities followed characteristic patterns in the cell line used. V-79 cells used as a non-malignant cell line, showed an increase in total Mg²⁺-ATPase activity per cell, especially in the EA less sensitive Mg²⁺-ATPase activity per cell. The physiological role of microsomal Mg²⁺-ATPase, however, was not clear; but, the microsomal EA less sensitive Mg²⁺-ATPase has been found to be insensitive to a variety of monovalent ions including Na⁺, K⁺ and Cl⁻, whereas the EA highly sensitive Mg²⁺-ATPase activity is affected by Cl⁻ or NO₃⁻ (7-9). These findings suggest that an ATP-Mg-requiring, ion-insensitive system operates during the confluent phase of V-79 cells.

In the V-79 cells used as a non-malignant cell line, showed an increase in total Mg²⁺-ATPase activity per cell, especially in the EA less sensitive Mg²⁺-ATPase activity per cell. The physiological role of microsomal Mg²⁺-ATPase, however, was not clear; but, the microsomal EA less sensitive Mg²⁺-ATPase has been found to be insensitive to a variety of monovalent ions including Na⁺, K⁺ and Cl⁻, whereas the EA highly sensitive Mg²⁺-ATPase activity is affected by Cl⁻ or NO₃⁻ (7-9). These findings suggest that an ATP-Mg-requiring, ion-insensitive system operates during the confluent phase of V-79 cells.

The increase in the microsomal protein concentration in the confluent phase of V-79 cells may explain the apparent decrease in the specific activities of the Na⁺, K⁺-, EA highly sensitive Mg²⁺- and Ca²⁺-ATPases that take place without any detectable change in the enzyme affinities for the substrate and cation cofactors. The reduced Na⁺, K⁺-ATPase activity per microsomal protein unit may represent reduced Na⁺, K⁺-ATPase activity per plasma membrane unit, and this may produce the reported decrease in K⁺ and Na⁺ translocating activity mediated by this enzyme (3, 10, 16). Analogously, the Ca²⁺ translocating activity in the plasma membrane of V-79 cells may decrease in the confluent phase. But, as microsomes consist of plasma membranes and other intracellular particles, changes in enzyme activities may result from changes in the microsomal membrane constituents and/or their enzyme activities.

By contrast, the HeLaS-3 cells used as malignant cells appear to have no change in their microsomal Na⁺, K⁺- and Mg²⁺-ATPases and protein concentrations. This probably is why there is a lack of change in the Na⁺ gradient across the plasma membrane and a lack of change in solute transport activities depending on the Na⁺ gradient, as reported previously (14). In contrast to the decreases in Na⁺, K⁺- and Mg²⁺-ATPase activities, a selective increase in microsomal Ca²⁺-ATPase activity took place in the confluent phase of HeLaS-3 cells. This is the first demonstration of such a density-dependent increase in microsomal Ca²⁺-ATPase activity. The importance of Ca²⁺ and Mg²⁺ and their interaction (4, 13) has been suggested; also,
one basic feature of cell malignancy is reportedly a defect in the regulation of intracellular Mg$^{2+}$ (13). The relation of the increase in microsomal Mg$^{2+}$-ATPase activity and intracellular Mg$^{2+}$ and Ca$^{2+}$ interactions to density-dependent growth regulation needs further analysis.

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