Comparative Properties of High Potassium and Low Potassium Sheep Erythrocyte Membrane Sodium-activated Adenosine Triphosphatase*

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SUMMARY

A comparative study of the Na+-stimulated adenosine triphosphatase system in high potassium (HK) and low potassium (LK) sheep red cells was carried out. For HK:LK activity, the approximate ratios were as follows: 10 for Na+-ATPase, 13 for Na+, K+-ATPase, and 2.7 for [14C]-ADP-ATP exchange activity; the ratio of Na+-stimulated membrane phosphorylation was approximately 7. With Na+ present, K+ at concentrations below 5 mM stimulated ATPase activity in HK membranes but not in LK membranes; above 5 mM, K+ inhibited Na+-ATPase, to an extent much greater in LK than HK membranes. [14C]ADP-ATP exchange in the presence of Na+ and oligomycin was similarly inhibited by K+, markedly in HK, but only slightly in HK membranes. These observations are consistent with data reported for the Na+, K+ pumping activity in HK and LK cells described by D. C. Tosteson (in G. A. JAMIESON AND T. J. GREENWALT (Editors), Red cell membrane, structure and function, J. B. Lippincott Company, Philadelphia, 1969, p. 291) and P. G. Hoffman (Ph.D. thesis, Duke University, 1969), and provide evidence at the molecular level for the physiological differences in activity between HK and LK cells.

It is well established that sheep have red cells possessing either low K+ or high K+ levels. This difference is a genetic trait, the LK type being dominant (2). Tosteson and Hoffman (8) have shown that the ouabain-sensitive active transport of Na+ and K+ operates 4 to 6 times faster in HK than in LK sheep red cells, and the ratio of rate constants for Na+ leakage to K+ leakage is 3 to 4 times higher in HK than in LK cells. These differences are manifest in the antigenic properties and in the Na+, K+-activated, ouabain-sensitive adenosine triphosphatase activity of the two types of cells. One antigen, M, is found in homozygous HK sheep cells, another, L, in homozygous LK cells (4-7); Na+, plus K+ -stimulated ATP hydrolysis was found to be 4- to 6-fold greater in HK than LK membrane preparations (8). The number of ouabain-binding sites is also higher in HK than in LK cells (9, 10).

Several studies have already provided evidence that Na+, K+-stimulated ATPase activity is associated with the Na+, K+-transport system (11, 12). It has been shown that Na+, K+ ATPase consists of a sequence of reactions including Na+-stimulated phosphorylation of a membrane component or intermediate (X) followed by hydrolysis of the phosphorylated intermediate (XP) (13-17). Accordingly, partial reactions ascribed to this ATPase system include Na+-stimulated [14C]ADP-ATP exchange (18, 19) and K+-activated phosphatase activity (20-24). The reaction sequence appears to involve also conformational alternations of the unphosphorylated and phosphorylated forms of the enzyme or intermediate, i.e. X1 = X2 and X1P = X2P (25-27).

It seemed apparent to us that if the differences in HK and LK red cells were due to differences in either (a) number of pumping sites or (b) qualitative or kinetic difference(s), or both, examination of the partial reactions involved in Na+ and K+ transport would provide direct evidence to distinguish these possibilities and shed some light on the nature of this genetic modification.

METHODS

Sheep blood was collected with heparin as anticoagulant (100 USP units per ml of blood), immediately stored at 0° to 4°, and used within 24 hours of collection. Whole blood potassium analyses were carried out with an Instrumentation Laboratory, Inc., flame photometer, model 143. Those samples with low K+ phenotype were assayed for the L antigen and M antigen. Homozygous and heterozygous LK preparations were designated as blood types LL and LM, respectively. Membranes were prepared following osmotic lysis of 0.9% NaCl-washed erythrocytes as described for human red cells (17), except that the post hemolytic residue was washed three times with 1 mM Tris-HCl containing 1 mM Tris-EDTA, pH 7.4, and then four times with 2 mM Tris-HCl, pH 7.4. Tris-EDTA (0.004 mM) was included in the final wash solution. The membrane residue was made up
to one-half the original packed cell volume in the final wash solution, and contained 2 to 4 mg of protein per ml. Membranes were stored at 4°C and used within 4 days after preparation.

The procedures for (a) labeling the membranes with γ-32P-ATP, (b) measuring ATPase activity as 32P, released from γ-32P-ATP, and (c) measuring [3H]ADP-ATP exchange activity were carried out as described previously (28). Phosphatase activity was determined as described by Robinson (29) with p-NPP as substrate. The incubation medium contained 50 mM Tris-HCl (pH 7.4), 4 mM MgCl₂, 4 mM Tris-p-NPP, and 1 to 2 mg of membrane protein per ml. NaCl and KCl were added as indicated. 32P-ADP, α-32P-ATP, and 8-3H-ATP were obtained from Schwarz BioResearch and p-NPP from Sigma.

Each experiment was carried out on a separate pair of membrane preparations, one from an LK and one from an HK sheep, and each determination was carried out in duplicate. Unless indicated otherwise the results reported are representative of at least four independent experiments.

Mg++-ATPase activity refers to the rate of ATP hydrolysis in the absence of added NaCl or KCl, Na+,-ATPase, to the increment in rate effected by added NaCl, and Na+, K+-ATPase, to the increment effected by added NaCl plus KCl.

RESULTS

With human red cell membranes, stimulation of ATPase activity by Na+ alone is observed at very low ATP concentrations (28). The effect of increasing concentrations of ATP on the stimulation of HK membrane ATPase activity by Na+ and by Na+ plus K+ is shown in Fig. 1. At the lowest concentration of ATP tested (0.05 μM) and with Na+ present, addition of K+ caused slight inhibition of ATPase. As the ATP concentration increased, hydrolysis of ATP was increasingly dependent on the presence of both Na+ and K+.

Membrane Phosphorylation—The detection and measurement of the relative amount of Na+-stimulated membrane phosphorylation associated with Na+,-stimulated ATPase activity were carried out with LK and HK sheep red cell membranes. The steady state level of 32P incorporated from γ-32P-ATP was measured with 2 μM γ-32P-ATP by means of brief incubation periods (10 s) as described previously (17). Preliminary experiments, particularly those at 0°C, showed that the level of bound 32P and its turnover rate, i.e., the rate of decrease of bound 32P following a MgATP or Tris-EDTA chase, were significantly greater than expected on the basis of Na+-ATPase. Accordingly, membrane phosphorylation in subsequent experiments was measured as the difference between the amount of 32P bound and 3H bound, with γ-32P[8-3H]ATP as substrate.

The amounts of 32P and 3H bound with 2 μM γ-32P[8-3H]ATP are shown in Fig. 2. In contrast to results obtained previously with human erythrocyte membranes (17), the amount of 3H-nucleotide bound was considerable relative to the amount of 32P bound. Identical amounts of bound nucleotide were obtained when a similar experiment was carried out with an additional set of tubes containing 2 μM α-32P-ATP. In view of these findings together with the observation that the ratio of Na+-ATPase to Na+,-ATPase activity is greater at ATP concentrations below 2 μM than at 2 μM, measurement of the Na+-dependent phosphorylation was carried out at 0.2 μM ATP.

In the presence of Na+ and 0.2 μM γ-32P[8-3H]ATP, the 3H bound was reduced relative to 32P bound (Fig. 3), the amount of 3H bound being approximately one-tenth the 32P bound. In view of these findings together with the observation that the ratio of Na+-ATPase to Na+-ATPase activity is greater at ATP concentrations below 2 μM than at 2 μM, measurement of the Na+-dependent phosphorylation was carried out at 0.2 μM ATP.
Na-ATPase of High and Low Potassium Sheep Erythrocyte Membranes

Fig. 2 (left). Membranes, 0.35 ml, were incubated in a final volume of 0.5 ml containing 2 mM γ-32P[8-3H]ATP, 12 mM MgCl₂, and 20 mM Tris HCl, pH 7.4. NaCl, 50 mM, and KCl, 10 mM, were added as indicated. 8-3H-ATP and γ-32P-ATP were added to give a final specific radioactivity of 2340 and 1170 cpm pmoles⁻¹, respectively. Incubation was for 10 s at 37°C. In the experiment shown, 32P release was determined in the trichloroacetic supernatant, and was 0.65, 257.4, and 357.9 pmoles min⁻¹ mg⁻¹ in HK membranes and 71.7, 109.5, and 75.0 pmoles min⁻¹ mg⁻¹ in LK membranes incubated without Na⁺ or K⁺ (C), with Na⁺, and with Na⁺ plus K⁺, respectively. The reaction was terminated and the radioactivity bound to the membranes was determined. The double label setting on the scintillation counter (Packard model 574) was adjusted so that no 3H counts appeared in the 32P channel. In view of the variability (±10%) of 32P in the 3H channel, approximately 20% of 32P counts appeared in the 3H channel. In view of the variability (±10%) of 32P in the 3H channel, approximately 20% of 32P counts appeared in the 3H channel. The radioactivity in the 3H channel was calculated from the amount of radioactive decay after a definite time interval, usually 2 weeks.

Values for membrane phosphorylation (pmoles mg⁻¹), calculated as the difference between the amount of 32P bound and 3H bound, are 0.00 without Na⁺ (C), 1.05 with Na⁺, and hence 0.15 for the Na⁺-stimulated increment in phosphorylation, for LK membranes. For HK membranes, the respective values are 0.65, 1.50, and 0.85.

Fig. 3 (center). Membranes, 0.35 ml, were incubated in a final volume of 0.5 ml as described in Fig. 2, except that the final γ-32P[8-3H]ATP concentration was 0.2 μM. Values for membrane phosphorylation (pmoles mg⁻¹), calculated as described in Fig. 2, are 0.00 without Na⁺ (C), 0.20 with Na⁺, and hence 0.12 for the Na⁺-stimulated phosphorylation, for LK membranes. For HK membranes, the respective values are 0.10, 0.88, and 0.03 pmoles mg⁻¹.

Fig. 4 (right). γ-3P-[γ-3H]ATP concentration was 0.2 μM. The data shown are calculated from six experiments which were carried out as described in Fig. 3.

### Table I

<table>
<thead>
<tr>
<th>HK No.</th>
<th>Activity</th>
<th>Experiment</th>
<th>HK (n=6)</th>
<th>LK (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na⁺-ATPase</td>
<td>Na⁺, K⁺-ATPase</td>
<td>pmoles ATP released/min</td>
<td>pmoles ATP released/min</td>
</tr>
<tr>
<td>238</td>
<td>50.6</td>
<td>95.1</td>
<td>3.1</td>
<td>6.3</td>
</tr>
<tr>
<td>72Y</td>
<td>80.7</td>
<td>102.7</td>
<td>6.1</td>
<td>7.1</td>
</tr>
<tr>
<td>18Z</td>
<td>93.3</td>
<td>118.5</td>
<td>6.8</td>
<td>9.9</td>
</tr>
<tr>
<td>76W</td>
<td>94.1</td>
<td>95.6</td>
<td>5.9</td>
<td>6.7</td>
</tr>
<tr>
<td>72Y</td>
<td>63.2</td>
<td>108.0</td>
<td>5.9</td>
<td>6.3</td>
</tr>
<tr>
<td>72Y</td>
<td>44.8</td>
<td>81.9</td>
<td>5.9</td>
<td>5.9</td>
</tr>
<tr>
<td>238</td>
<td>63.4</td>
<td>95.6</td>
<td>5.4</td>
<td>7.4</td>
</tr>
<tr>
<td>Mean ± S.E.</td>
<td>69.2 ± 110.6</td>
<td>110.6 ± 35.3</td>
<td>8.8 ± 8.5</td>
<td>3.0 ± 2.8</td>
</tr>
</tbody>
</table>

Membranes. The Na⁺-stimulated component in HK membranes was thus approximately 7 times that in LK membranes, whereas the unstimulated levels were comparable.

**Na⁺-ATPase and Na⁺, K⁺-ATPase—** A comparison of the relative Na⁺-ATPase and Na⁺, K⁺-ATPase activities in LK and HK membranes was carried out with 0.2 μM ATP. The conditions were similar to those for the labeling experiments except that longer incubation periods were used to obtain greater accuracy. As shown in Table I, the average values for Na⁺-ATPase and Na⁺, K⁺-ATPase of HK membranes were 10- and 15-fold the values obtained for LK membranes.

**Na⁺-Stimulated [³⁴C]ADP-ATP Exchange at 0°C—** To determine whether the rate of an initial phosphorylation step is also lower in LK than HK membranes, [³⁴C]ADP-ATP exchange activities were measured with 2 μM ATP and 1 μM [³⁴C]ADP (28). The assays were carried out at 0°C to minimize ATP hydrolysis and hence changes in ATP and ADP concentrations. At this temperature, the maximal amount of ATP hydrolyzed was less than 2%, i.e. in HK membrane incubated with Mg²⁺ plus Na⁺. Oligomycin was included as indicated in Table II since several studies have indicated that this inhibitor acts at a step after initial transphosphorylation (25, 31, 32); with human red cell membranes this effect was manifested in a marked stimulation of exchange by oligomycin, presumably due to inhibition of hydrolysis of phosphorylated intermediate (28). The results of six experiments are shown in Table II. Na⁺-Stimulated exchange refers to the activity observed in the presence of 50 mM NaCl after subtraction of the activity obtained with 50 mM KCl.
The data shown are from Experiments 1 and 6 of Table II. Values shown are the means of triplicate samples.

### Table II

**Comparison of Na⁺-stimulated [³⁴C]ADP-ATP exchange activity in LK and HK membranes at 0°**

Membranes (1 ml) were initially incubated with either 15 μl of ethanol (minus oligomycin) or 15 μl of oligomycin (5.5 mg per ml of ethanol, plus oligomycin). After 20 min at 37°, the mixtures were diluted with 2 volumes of 2 mM Tris-HCl in 0.004 mM Tris-EDTA, pH 7.4. Initial incubation was continued for 10 min at 0° and then 0.1 ml was added to 0.15 ml of reaction medium. The final incubation medium contained 2 μM Tris-ATP, 1 μM [³⁴C]ADP (0.025 μCi/0.25-ml volume), 12 μM MgCl₂, and either 50 mM NaCl or 50 mM KCl. Final membrane protein concentration was 0.24 to 0.4 mg per ml. Incubation was carried out for 1 or 12 hours at 0°. The reaction was terminated and analyses were performed as described previously (26). Na⁺-Stimulated exchange refers to the activity observed in the presence of 50 mM NaCl after subtracting the activity observed in the presence of 50 mM KCl. Experiments 1 and 5 were carried out on the same preparations of LK and HK membranes.

<table>
<thead>
<tr>
<th>HK No.</th>
<th>Na⁺-Stimulated exchange</th>
<th>Na⁺-Stimulated exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minus oligomycin</td>
<td>Plus oligomycin</td>
</tr>
<tr>
<td></td>
<td>pmoles/mg/hr</td>
<td>pmoles/mg/hr</td>
</tr>
<tr>
<td>76W</td>
<td>51.9 443.7</td>
<td>259 LL</td>
</tr>
<tr>
<td>72Y</td>
<td>20.1 216.8</td>
<td>241 LL</td>
</tr>
<tr>
<td>72Y</td>
<td>107.5 365.6</td>
<td>259 LL</td>
</tr>
<tr>
<td>72Y</td>
<td>28.3 218.1</td>
<td>241 LL</td>
</tr>
<tr>
<td>72Y</td>
<td>57.0 216.9</td>
<td>241 LL</td>
</tr>
<tr>
<td>51X</td>
<td>112.0 414.6</td>
<td>277 LL</td>
</tr>
<tr>
<td>Mean</td>
<td>303.0 ± 114.6</td>
<td>36.5 ± 36.5</td>
</tr>
</tbody>
</table>

### Table III

**Effect of K⁺ on Na⁺-dependent [³⁴C]ADP-ATP exchange**

The data shown are from Experiments 1 and 6 of Table II. Values shown are the means of triplicate samples.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>[³⁴C]ADP-ATP exchange activity</th>
<th>Percentages of inhibition by K⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 mM KCl</td>
<td>50 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>pmoles/mg/hr</td>
<td>pmoles/mg/hr</td>
</tr>
<tr>
<td>LK No. 277  ( LL) . . . . .</td>
<td>7.2 19.2</td>
<td>19.5 89.7</td>
</tr>
<tr>
<td>HK No. 51X . . . . . . . . .</td>
<td>10.1 57.8</td>
<td>122.2 413.7</td>
</tr>
<tr>
<td>HK No. 259 ( LL) . . . . . .</td>
<td>41.3 63.8</td>
<td>488.0 347.3</td>
</tr>
</tbody>
</table>

In the absence of oligomycin, Na⁺-stimulated exchange in homozygous LK membranes was generally lower than in HK membranes, but the variation in activity among preparations was too large for meaningful comparison. Following prior incubation with oligomycin, Na⁺-stimulated exchange was increased both in HK and LK membranes. Under this condition of minimal ATP hydrolysis, the HK: LK Na⁺-stimulated exchange ratio was approximately 2.7. Although there was considerable quantitative variation in measurements obtained with the different preparations, the average deviation from the mean of duplicate or triplicate determinations was less than 5%.

**Effects of K⁺ on Na⁺-Stimulated [³⁴C]ADP-ATP Exchange and on Na⁺-ATPase**—In view of the known asymmetry of the Na⁺, K⁺ pump and ATPase system, intracellular Na⁺ should stimulate [³⁴C]ADP-ATP exchange. Since intracellular K⁺ inhibits the K⁺ pump at lower concentrations in LK than HK membranes (33, 34), it was of interest to test the relative effects of K⁺ on the exchange reaction. Two experiments with 10 mM KCl in addition to Na⁺ and oligomycin are shown in Table III. As indicated, the Na⁺-stimulated exchange was inhibited in LK membranes to a much greater extent than in HK membranes, i.e. 65% compared to 2% in one experiment and 75% compared to 31% in the other.

An analysis of the effect of K⁺ on Na⁺-ATPase was carried out at 0.2 μM ATP as shown in Fig. 5. In the absence of added Na⁺, the percentage inhibition of ATPase activity by K⁺ was very small. In the presence of Na⁺, addition of 1 mM K⁺ increased Na⁺-activated hydrolysis 25% in HK membranes (cf. Table I); inhibition was observed with higher K⁺ levels. In contrast with HK membranes little stimulation of ATPase activity occurred in the presence of the lowest levels of K⁺ (0.05 to 1 mM).

Increasing concentrations were more inhibitory to Na⁺-ATPase activity in LK than HK membranes. Identical K⁺ inhibition was observed in one experiment with heterozygous (LM) LK membranes (not shown). The K⁺ concentrations effecting 50% inhibition were approximately 3 mM and >20 mM for LK and HK membranes, respectively.

**K⁺-Activated Phosphatase**—K⁺-Activated, ouabain-sensitive membrane phosphatase activity, ascribed to be a terminal step in the Na⁺, K⁺-ATPase reaction sequence, has been measured by using synthetic substrates such as p-NPP (20-24, 29). We have attempted to measure and compare this activity in membranes prepared from HK and LK sheep red cells. Under the conditions described in Table IV, the activity observed with 4 mM p-NPP
TABLE IV

Test for K\textsuperscript{+}-phosphatase activity in LK and HK membranes

Membranes, 0.1 ml, were incubated in a final volume of 0.25 ml containing 4 mM p-NPP, 4 mM MgCl\textsubscript{2}, 30 mM Tris-HCl, pH 7.4, and KCl as indicated. Incubation was for 30, 45, or 60 min at 37\textdegree. The reaction was terminated with 0.25 ml 10% trichloroacetic acid. Following centrifugation, 0.2 ml of supernatant was added to 1.0 ml of 1 M Tris and the optical density at 420 nm was measured as described by Robinson (29). The values shown are the mean \pm S.E. of the number of separate experiments shown in parentheses. The results for human membranes is representative of six experiments.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>p-NPP-Phosphatase activity</th>
<th>Stimulation by K\textsuperscript{+}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mM KCl</td>
<td>10 mM KCl</td>
</tr>
<tr>
<td>LK</td>
<td>0.15 \pm 0.02 (4)</td>
<td>0.15 \pm 0.30 (4)</td>
</tr>
<tr>
<td>HK</td>
<td>0.21 \pm 0.01 (4)</td>
<td>0.22 \pm 0.02 (4)</td>
</tr>
<tr>
<td>Human</td>
<td>0.20</td>
<td>0.33</td>
</tr>
</tbody>
</table>

...and 4 mM MgCl\textsubscript{2}, although 40% higher in HK than LK membranes, was not affected by the addition of KCl (10 mM). This is in contrast to the activating effects of K\textsuperscript{+} on the phosphatase activity of human membranes described by others (20, 35, 36) and shown here for comparative purposes. Other experiments (not shown) indicated that the activity was unaffected or decreased by Na\textsuperscript{+}, and addition of K\textsuperscript{+} in the range of 0.1 to 10 mM did not increase the activity observed with Mg\textsuperscript{2+} alone.

**DISCUSSION**

The data presented in this report indicate that the quantitive differences in the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase activity in HK and LK sheep red cell membranes are evident in the partial reactions of components associated with transport ATPase. The steady state level of phosphorylated intermediate was approximately 7-fold greater in HK than LK membranes. This difference is consistent with the ratio of ouabain-binding sites in HK compared with LK red cells measured by Dunham and Hoffman (9, 10). They showed that the approximate number of 3H-ouabain-binding sites was 44 in HK cells and 6 in LK cells. Since phosphorylation of the intermediate was practically maximal at 0.2 \mu M ATP (compare the values for the Na\textsuperscript{+}-stimulated increment in 35S bound given in the legends and calculated from the data in Figs. 2 and 3), an estimate of the number of phosphorylation sites per cell can be calculated from the data in Fig. 4; with 2 mg of membrane protein equivalent to \sim 2.0 \times 10\textsuperscript{8} cells, the number of sites per cell is approximately 42 and 6 for HK and LK cells, respectively.

Kinetic differences between HK and LK red cell membrane Na\textsuperscript{+}-ATPase and Na\textsuperscript{+}-stimulated [\textsuperscript{32}P]ADP-ATP exchange were manifested by differences in response (inhibition) to increasing K\textsuperscript{+} levels. The much greater sensitivity of LK Na\textsuperscript{+}-ATPase to K\textsuperscript{+} inhibition correlates closely with differences in effects of K\textsuperscript{+} on the Na\textsuperscript{+}-K\textsuperscript{+} pump, described by Tosteson (33) and later by P. G. Hoffman (34). They showed that intracellular K\textsuperscript{+} inhibits the pump at much lower concentrations in HK than LK cells.

Since external K\textsuperscript{+} ions stimulate (37, 38) and internal K\textsuperscript{+} ions inhibit the pump and the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase, particularly in sheep red cells (33, 34), Tosteson has emphasized that estimates of the maximum velocity of the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase would be erroneous when measured in broken membrane preparations. Accordingly, it might be expected that a truer estimate of the HK:LK Na\textsuperscript{+}, K\textsuperscript{+}-ATPase activity ratio would be lower than the reported values of 4 to 6 (8). In our experiments, in which the Na\textsuperscript{+}-stimulated component of ATPase was apparent at very low ATP concentrations, the HK:LK ratios of Na\textsuperscript{+}-ATPase and Na\textsuperscript{+}, K\textsuperscript{+}-ATPase were actually relatively high, i.e. 10 and 13, respectively. However, this Na\textsuperscript{+}-ATPase activity ratio is similar to the ratio of pump activities observed by Tosteson (33) and P. G. Hoffman (34) under conditions of very low intracellular K\textsuperscript{+}.

It has been suggested (39) that Na\textsuperscript{+}-ATPase measured at low concentrations of ATP is distinct from Na\textsuperscript{+}, K\textsuperscript{+}-ATPase measured at high concentrations of ATP and associated with Na\textsuperscript{+} and K\textsuperscript{+} transport. Our data support the hypothesis that the activities are both measures of the same multistage reaction sequence. Thus the relative activities of the partial reactions observed at micromolar ATP concentrations were low in LK red cells having low intracellular K\textsuperscript{+} levels and low K\textsuperscript{+} pump activity and high in HK cells which have relatively high pump activity. Moreover, the relative inhibitory effects of K\textsuperscript{+} on Na\textsuperscript{+}-ATPase are strikingly similar to the effects of intracellular K\textsuperscript{+} on the K\textsuperscript{+} pump in the two types of cells. It is likely that different reaction steps become rate-limiting as the ATP concentration is changed, i.e. as the ATP concentration is decreased, Na\textsuperscript{+}-stimulated step(s) preceding K\textsuperscript{+}-activated hydrolysis of phosphorylated intermediate become rate-limiting, as discussed previously (28).

If the difference between LK and HK cells is simply the number of pump sites, the HK:LK activity ratio should be constant irrespective of the partial reaction measured, substrate level, or temperature. When [\textsuperscript{14}C]ADP-ATP exchange activity was determined under the conditions of minimal ATP hydrolysis (0\textdegree in the presence of oligomycin), the exchange rate in HK membranes was only \sim 2.7 times that of LK membranes in contrast to the quantitatively greater differences in Na\textsuperscript{+}-ATPase and Na\textsuperscript{+}, K\textsuperscript{+}-ATPase activities. When these observations are considered in terms of a plausible sequence of reaction steps of the Na\textsuperscript{+}, K\textsuperscript{+}-ATPase system (14-17, 28), i.e.

\[
E_1 \underset{k_{-1}}{\overset{k_1}{\rightarrow}} E_1 \cdot ATP \underset{k_{-2}}{\overset{k_2}{\rightarrow}} E_1P \cdot ADP \underset{k_{-3}}{\overset{k_3}{\rightarrow}} E_1P \\
E_1P \underset{k_{-4}}{\overset{k_4}{\rightarrow}} E_1P \underset{k_{-5}}{\overset{k_5}{\rightarrow}} E_1P \underset{k_{-6}}{\overset{k_6}{\rightarrow}} (E_1) 
\]

it is possible that the rate-limiting step for Na\textsuperscript{+}-ATPase at 37\textdegree is subsequent to initial transphosphorylation, i.e. at the step \textit{E1P} \rightarrow \textit{E1P}. Although there may be fewer pump sites in LK than in HK cells, our data indicate that this cannot be the basis, or at least the only basis, for the difference in the two types of cells. A difference in the kinetic characteristics of the sites is suggested from the data; a difference in a single rate constant as discussed by P. G. Hoffman (34), for example \textit{k3}, in the scheme depicted here, would be expressed in the relative proportion of forms of phosphorylated intermediate, e.g. \textit{E1P} \rightarrow \textit{E1P}, and also the number of pump sites in the conformation which could bind ouabain \textit{e.g.} outward facing form. If the steady state level of \textit{E1P} = \textit{E1P}

\footnote{Release of ADP and Pi at different steps has been suggested by Garrahman and Glynn from studies on Na\textsuperscript{+}-Na\textsuperscript{+} exchange (40) and in previous studies on the effects of oligomycin on membrane Na\textsuperscript{+}-ATPase (28).}
is greater in HK than LK cells, the greater difference in over-all Na+-ATPase activity compared to exchange activity could be expected. Similarly, if K+ interaction with inward facing form(s) inhibits their turnover, the lower sensitivity of HK than LK membranes to inhibition by K+ would be a plausible consequence of a greater $E_2P:E_1P$ steady state ratio. It is further possible that the foregoing effects of Kf and the greater K+ inhibition at low ATP have a common kinetic explanation.

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