Studies of High Potassium and Low Potassium Sheep Erythrocyte Membrane Sodium-Adenosine Triphosphatase

INTERACTIONS WITH OLIGOMYCYIN, ADENOSINE TRIPHOSPHATE, SODIUM, AND POTASSIUM*

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SUMMARY

The effects of oligomycin on Na+ATPase of high K (HK) and low K (LK) sheep erythrocyte membranes have been investigated. Activation of LK Na+ATPase is observed with ATP ≤ 0.2 μM; activation of HK is observed with ATP ≤ 0.02 μM. Inhibition occurs with higher ATP. At 0.2 μM ATP, oligomycin stimulation of LK Na+ATPase is associated with a 3- to 4-fold increase in the 32P intermediary; inhibition of HK is associated with only a slight increase (1.3-fold) in 32P intermediary. The effects of oligomycin are similar for HK and LK in that activation occurs at low catalytic rates (≤20 pmoles mg⁻¹ min⁻¹); inhibition occurs at higher rates, irrespective of the means of altering the rate (varying ATP or Na⁺ concentration, or both; stimulation of HK with specific isoimmune antiserum). Oligomycin counteracts K⁺ inhibition (LK) and K⁺ counteracts oligomycin inhibition (HK). The results are consistent with a reaction sequence involving oligomycin-sensitive conformational changes of phosphorylated and probably unphosphorylated intermediate, i.e. EᵢP → EᵢP and Eᵢ → Eᵢ; the resulting shift in equilibrium can, at low catalytic rates, be evidenced in stimulation of Na⁺-ATPase.

Interaction of HK and LK Na⁺ATPase with Na⁺, K⁺, and ATP are interdependent and markedly different for the two; at constant ATP (0.2 μM), HK is more sensitive to activation by Na⁺ and less sensitive to inhibition by K⁺ than LK ATPase. Although effects of both K⁺ and oligomycin are dependent on ATP concentration, a difference in affinity for ATP in addition to, or as a result of, a different relative affinity for Na⁺ and K⁺ may be the basis for the distinctions between HK and LK membranes.

Erythrocyte membranes have certain advantages for studies of the enzymic basis of ion transport. These include (a) the occurrence of a genetic modification of the ion transport system in red cells of certain species, e.g. high potassium (HK) and low potassium (LK) sheep erythrocytes (2), (b) the evidence in red cells for a close relationship between ion transport and membrane-bound Na⁺-activated ATPase activity (3-6), and (c) the relative purity of mammalian erythrocyte membranes compared to many other membrane preparations.

Previous studies showed that Na⁺K⁺-ATPase activity of HK and LK sheep red cells correlated quantitatively with the Na⁺K⁺-pump activity of the two types of cells (7, 8), i.e. both activities were several-fold higher in HK cells as compared to LK cells. A similar difference was also apparent in the partial reactions associated with "transport ATPase" (9). The steady state level of Na⁺-dependent membrane phosphorylation (phosphorylated intermediate) was approximately 7-fold higher in HK than LK membranes; a similar ratio was found for the number of ouabain-binding sites in the two types of cells (10).

In addition to the quantitative difference between HK and LK membrane Na⁺ATPase, kinetic differences were also found when the system was studied at low ATP concentration (9). In particular, the two types of membranes differed in their response to K⁺; the HK type was activated by low levels of K⁺ and inhibited only with relatively high concentrations, whereas the LK type was markedly inhibited by K⁺, even at low concentrations.

In the present study we have continued the investigation of these quantitative and kinetic differences to elucidate further the nature of this genetic modification which may be of fundamental importance in understanding the Na⁺,K⁺-ATPase enzyme system.

METHODS

Membranes were prepared from fresh, sheep erythrocytes as described previously (9). The volume of the suspensions, equivalent to one-half of the original packed cell volume, contained 2 to 4 mg of protein per ml; further dilutions were done as indicated, with 2 mM Tris-HCl (pH 7.4). Procedures for labeling membranes with 32P using [8-³²P]ATP and for

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measuring ATPase activity as \(^{32}\text{P}\) released from \([\gamma-^{32}\text{P}]\text{ATP}\) were carried out, as described earlier (9), in a medium containing various amounts of \([\gamma-^{32}\text{P}]\text{ATP}\), 12 \(\mu\text{M}\) MgCl\(_2\), and 30 \(\text{mM}\) Tris-HCl (pH 7.4). Unless indicated otherwise, treatment with oligomycin was carried out by preincubating 6 volumes of diluted membranes (1 to 2 mg of protein per ml) with 0.05 volume of absolute ethanol containing 6 mg of oligomycin per ml (9). Control tubes, without OM, were preincubated with 0.05 volume of ethanol alone. After 20 min of preincubation, an aliquot of the membrane suspension was added to the appropriate volume of absolute ethanol containing 6 mg of 

indicated, with MgCl\(_2\) plus 50 m\(\mu\)l Tris-HCl. Unless indicated otherwise, the results reported are representative of replicate independent experiments.

Oligomycin (approximately 15% oligomycin A and 85% oligomycin B) and Tris ATP were purchased from Sigma. \([\gamma-^{32}\text{P}]\text{ATP}\) was prepared as described (11) and \([8-^{3}\text{H}]\text{ATP}\) was obtained from Schwarz-BioResearch.

Five HK and five LK sheep were used. Most of the experiments were carried out with three of the LK sheep which were homozygous for the L antigen; differences in parameters measured in homozygous and heterozygous LK sheep membranes could not be detected.

RESULTS

Effects of Oligomycin—Previous experiments with HK and LK red cell membranes showed that their \(\text{Na}^+\)-dependent \([^{14}\text{C}]\text{ADP-ATP}\) exchange rates with oligomycin present were more similar in magnitude than the \(\text{Na}^+\)-dependent ATP hydrolysis rates; i.e. the HK:LK activity ratio for exchange was 2.7, whereas that for hydrolysis was approximately 10. Moreover, these studies (9) and those of others (12-17) suggested that oligomycin inhibits the multistage \(\text{Na}^+\)-ATPase at a step other than the sodium-stimulated phosphorylation of an "intermediate." In the present study, we examined further the effects of oligomycin on the two types of membranes to determine whether their difference is reflected in a difference in rate of a step prior to, or subsequent to, the step affected by oligomycin.

The effects of oligomycin on the \(\text{Na}^+\)-stimulated phosphorylation (\(^{32}\text{P}\)-intermediate) are shown in Fig. 1. At 0.2 \(\mu\text{M}\) ATP, oligomycin markedly increased the \(^{32}\text{P}\) bound in LK (3- to 4-fold), but only slightly increased that bound in HK. At lower ATP (0.02 \(\mu\text{M}\)), oligomycin increased phosphorylation in HK as well as in LK, but to a lesser extent (~1.7-fold). To determine whether these effects represented increased or decreased catalytic center activity, the \(\text{Na}^+\)-ATPase rate was also measured under identical conditions. The \(\text{Na}^+\)-ATPase divided by the \(\text{Na}^+\)-stimulated increment in phosphorylation, i.e., the turnover or catalytic center activity is shown in Fig. 2 for experiments carried out at 0.2 \(\mu\text{M}\) ATP. In the absence of oligomycin, the catalytic center activity of HK was about 3-fold that of LK. In the presence of oligomycin, the turnover of HK was decreased and that of LK was not changed, with the result that the turnover of the two types in the presence of oligomycin tended to be similar.

Oligomycin increased the level of phosphorylated intermediate in LK membranes (Fig. 1) and did not affect its turnover (Fig. 2) due to a stimulation of \(\text{Na}^+\)-ATPase activity. This is shown in Fig. 3 which includes experiments carried out with various ATP concentrations, both lower and higher than those depicted in Fig. 1.
Figs. 1 and 2. In Fig. 3 the Na\(^+\)-ATPase activity without oligomycin is the control and is represented as 100%. Values of Na\(^+\)-ATPase with oligomycin are represented as a percentage of the control; i.e., values above the 100% line represent stimulation, and values below represent inhibition. At 0.2 \(\mu\)M ATP (cf. Fig. 2), inhibition of HK and stimulation of LK Na\(^+\)-ATPase were observed. However, the curves for both systems were similar with respect to the apparent stimulation observed at the lowest ATP concentrations followed by a tendency toward inhibition at higher ATP concentrations. Similar results were obtained with higher Mg\(^2+\) concentrations (0.1 mM). Activating effects could not be observed by varying the concentrations of oligomycin.

When the Na\(^+\) concentration was varied, it was also found that the oligomycin response could be altered. This may reflect an influence of the inhibitor on the kinetics of Na\(^+\) activation, as described by Robinson (17) and shown in Fig. 4. In the absence of oligomycin, the Na\(^+\) activation curve for HK membranes approximates Michaelis-Menten kinetics and is markedly different from that obtained with LK, e.g., 20 mM Na\(^+\) markedly stimulated HK but failed to increase the activity of LK above that observed without added Na\(^+\). Furthermore, the activity of LK is lowered by the addition of small amounts of Na\(^+\) (≤5 mM) to values below that observed without added Na\(^+\). If the activities are compared to the activity without added Na\(^+\) but with 50 mM K\(^+\) added to counteract possible activation by trace amounts of Na\(^+\), the Na\(^+\)-response curve appears to consist of two components; one is activated by low levels of Na\(^+\), whereas the other is activated by >20 mM Na\(^+\).

With oligomycin present, the sensitivity to activation by Na\(^+\) was markedly increased in both HK and LK membranes. The data in Fig. 4 also show that without added Na\(^+\), oligomycin stimulates ATPase activity, particularly in HK membranes. This stimulatory effect was abolished when 50 mM KCl was included in the medium to obliterle effects of small amounts of Na\(^+\).

It has been shown previously that oligomycin inhibits K\(^+\)-stimulated hydrolysis of the \(\beta\)P-intermediate (11, 12). The interaction of oligomycin and K\(^+\) with the membrane Na\(^+\)-ATPase assayed at 0.2 \(\mu\)M ATP and 50 mM NaCl are shown in Table I. In the absence of oligomycin, 5 mM KCl stimulated HK and inhibited LK Na\(^+\)-ATPase in accord with previous observations (9). With HK membranes, K\(^+\) tended to counteract oligomycin inhibition, i.e., the percentage inhibition was less with K\(^+\) present; with LK membranes, K\(^+\) inhibition was counteracted by oligomycin. These results are compatible with inhibition by oligomycin at a step in which there is a transformation of an intermediate or site to a K\(^+\)-sensitive state, whether the K\(^+\) effect is inhibitory or stimulatory.

Interaction with ATP, Na\(^+\), and K\(^+\)—The foregoing results have shown that the oligomycin response is dependent on the ATP and, to an extent, the Na\(^+\) concentrations and is counteracted by K\(^+\). Furthermore, interaction of membranes with ATP (ATP binding) is decreased by K\(^+\) (18, 19), an effect counteracted by Na\(^+\). These observations have raised the possibility that the basis for the differences between HK and LK transport systems is their relative affinities for ATP which in turn affect the \(K_m\) for Na\(^+\) and K\(^+\) or vice versa, or both.

Michaelis constants (\(K_{\text{ATP}}\)) for Na\(^+\)-ATPase were determined with a range of ATP concentrations from 0.01 to 2 \(\mu\)M. Although the values obtained were similar to the reported dissociation constant for ATP binding (18, 19), variation in values determined with preparations from different animals precluded a meaningful comparison of HK and LK; \(K_{\text{ATP}}\) for LK was 0.13 ± 0.04 \(\mu\)M (three animals), whereas for HK it was 0.06 ± 0.02 \(\mu\)M (four animals). However, the response of Na\(^+\)-ATPase to K\(^+\) was found to be dependent on ATP concentration, particularly with HK membranes, and to be different for the two types of membranes as shown in Fig. 5. As the ATP concentration increased, stimulatory effects of K\(^+\) decreased and inhibitory effects increased. Nevertheless, with the ATP concentration varied by two orders of magnitude, the two cell types remained different, i.e., the K\(^+\) response of HK measured at 2 \(\mu\)M ATP was distinct from that of HK measured at 0.02 \(\mu\)M.

When the Na\(^+\) concentration was varied, the response to K\(^+\) was altered as shown in Fig. 6. Decreasing the Na\(^+\) increased inhibition by K\(^+\) in both HK and LK membranes. The interaction of both activating and inhibitory effects of K\(^+\) and the action of Na\(^+\) on these effects are also apparent with HK membranes. Na\(^+\) not only decreased K\(^+\) inhibition as described above, but it also decreased K\(^+\) activation as indicated by the decrease in K\(^+\) activation (1 mM KCl) as Na\(^+\) was increased from 5 to 20 mM. With 1 mM Na\(^+\), the response pattern for HK tended to resemble that of LK measured with 20 mM Na\(^+\). When similar data are plotted as activity versus Na\(^+\) concentration (Fig. 7), an apparent decrease in activity at low Na\(^+\) levels is evident in HK in the absence of K\(^+\) and is more marked.

### TABLE I

**Interaction of oligomycin and K\(^+\) with Na\(^+\)-ATPase of HK and LK membranes**

Assays were carried out at 0.2 \(\mu\)M \([\gamma\text{-}\text{P}]\)ATP as described in Fig. 3.

| Group type | Additions | Activity (\(\text{pmoles ATP released/min/mg}\)) |
|------------|-----------|----------------------------------------------|
|            | Without oligomycin | With oligomycin |
| HK         | None      | 17.2 | 33.3 |
|            | 50 mM NaCl | 84.2 | 48.1 |
|            | 50 mM NaCl + 5 mM KCl | 126.5 | 108.3 |
| LK         | None      | 9.4  | 19.8 |
|            | 50 mM NaCl | 18.3 | 29.2 |
|            | 50 mM NaCl + 5 mM KCl | 9.6 | 22.9 |
FIG. 5. Effect of ATP concentration on K+ response of HK and LK Na+-ATPase. Na+-ATPase assays were carried out in a final volume of 0.25 ml containing 0.1 ml of membranes, 12 μM MgCl₂, 30 mM Tris-HCl (pH 7.4), and various amounts of 32P-ATP as indicated. HK membranes (diluted, 1.02 mg of protein per ml) were incubated at 37° for 0.5, 2, and 4 min at ATP concentrations of 0.02, 0.20, and 2.0 μM, respectively. LK membranes (diluted, 0.97 mg per ml) were incubated at 37° for 6 and 10 min at ATP concentrations of 0.2 and 2.0 μM, respectively. Na+-ATPase activity was calculated by subtracting activities measured with 50 mM KCl from activities measured with 50 mM NaCl plus the amounts of added KCl indicated.

FIG. 6. Effect of Na+ concentration on the responses of HK and LK Na+-ATPase to K+. ATPase activity was determined at 0.2 μM ATP as described in Fig. 5, with HK and LK membranes diluted to contain 0.66 and 1.0 mg of protein per ml, respectively. LK membranes: •••••, without added K+; O---O, 5 mM KCl; ▼------▼, 5 mM KCl. Right, HK membranes: •••••, without added K+; O---O, 5 mM KCl; ▼------▼, 20 mM KCl.

TABLE II

| Serum                  | Na+-ATPase activity (gmoles/min/mg protein) | Ratio of b2/a |
|------------------------|---------------------------------------------|---------------|
|                         | Without oligomycin (a)                      | With oligomycin (b) |
| Lyophilized nonimmune  | 3.3 ± 0.8                                  | 9.8 ± 3.1     |
| Anti-L                 | 22.6 ± 6.0                                | 11.6 ± 2.9    |
|                         | 140.0                                      | 76.9          |

**DISCUSSION**

A considerable amount of evidence from a number of studies suggests that the Na⁺, K⁺-ATPase reaction sequence consists not only of a Na⁺-activated phosphorylation of some membrane component(s) and a K⁺-activated dephosphorylation (23), but also includes transitions of the phosphorylated and unphosphorylated forms of the intermediate (11-13, 24), i.e., $E_1 \rightarrow E_1P \rightarrow E_2P \rightarrow E_0 \rightarrow (E_0)$, as depicted in Fig. 8. The model includes interaction of K⁺ with $E_0$ and $E_2P$ which can account for K⁺ effecting both inhibition (removal of $E_0$) and hydrolysis, respectively. Oligomycin, by shifting the equilibrium from $E_2P$ to $E_2$, probably $E_0$ to $E_1$, counteracts interaction of K⁺.
FIG. 8. The upper scheme illustrates plausible reactions involved in Na+-ATPase. $E_1$ and $E_0$ represent two forms of unphosphorylated intermediate, and $E_P$ and $E_0P$ represent two forms of phosphorylated intermediate. $A$ refers to ATPase activity via hydrolysis of $E_P$, and $B$ refers to ATPase activity via the sequence $E_0P \rightarrow E_0 + P$. The lower scheme illustrates interaction of identical active subunits which, in turn, may condition the interaction of the system with substrate and effectors.

with the system and vice versa, and increases the apparent affinity for Na$^+$, as observed in these studies.

Decreased inhibition of Na$^+$, K$^+$-ATPase by oligomycin as the ATP concentration is decreased has been observed by Robinson (17). He suggested two possible routes of hydrolysis: hydrolysis of $E_P$ (low $T_{\text{max}}$, Pathway A of Fig. 8) and hydrolysis of $E_0P$ (high $T_{\text{max}}$, Pathway B of Fig. 8). His model does not necessarily include $E_1 \rightarrow E_0$. At low ATP concentration, hydrolysis of $E_P$ could be relatively faster than that of $E_0P$, but the over-all rate limited by rapid conversion of $E_1P$ to $E_0P$. With oligomycin, the equilibrium would be shifted towards $E_1$ forms; Pathway B would be decreased, and Pathway A would still function and appear as a stimulation, particularly if interaction of ATP with $E_1$ is no longer decreased by the equilibrium $E_1$ with $E_0$. Other possibilities cannot be ruled out; in particular, if oligomycin does not inhibit completely $E_1P \rightarrow E_0P$ (and $E_1 \rightarrow E_0$), a concomitant increase in initial phosphorylation ($E_1P \rightarrow E_1P$) would be manifested in either activation or inhibition depending upon whether the transition step ($E_1P \rightarrow E_0P$) is relatively faster or slower than the phosphorylation step, respectively.

That the effects of oligomycin are dependent on the absolute activity, per se, are indicated when the effects are examined as shown in Fig. 9. Here all values for Na$^+$-ATPase activity obtained at various ATP concentrations and at constant Na$^+$ concentration (50 mM) are plotted. The Na$^+$-ATPase in the presence of oligomycin is shown as a percentage of the control. The open symbols represent LK sheep (each symbol represents a different animal), and the closed symbols represent HK sheep. As shown, there is considerable overlap of the values of HK and LK. The data are depicted in this manner in order to emphasize that when the absolute Na$^+$-ATPase activity is greater than about 20 pmoles mg$^{-1}$ min$^{-1}$, the effect of oligomycin is inhibitory. At lower activity rates, oligomycin stimulates Na$^+$-ATPase. The dependence of the oligomycin effect on the absolute activity is apparent not only when the activity is altered by changes in substrate and activator (Na$^+$) concentrations, but also when the activity is altered (increased) by the specific reaction of LK-type membranes with specific isoimmune serum as shown in Table II.

FIG. 9. Effects of oligomycin on Na$^+$-ATPase of HK and LK membranes. Each symbol represents the activity, determined in a separate experiment, of an HK (closed symbols) or an LK (open symbols) sheep; each different symbol represents a different animal. Values are taken from experiments carried out at various ATP concentrations as described in Fig. 3.

ATP concentration affected the response of Na$^+$-ATPase not only to oligomycin but also to K$^+$; however, the K$^+$-response pattern for LK remained different from that for HK membranes, even when the ATP concentration was varied by two orders of magnitude. It is plausible that a difference in affinity for ATP, in addition to or as a result of different affinities for Na$^+$ and K$^+$, is a basic kinetic distinction between the two types of membrane Na$^+$-ATPase and Na$^+$, K$^+$-pump systems. When a constant ATP concentration (0.2 $\mu$M) was varied, the K$^+$ response of HK resembled that of LK when the Na:K concentration ratio for HK was 1/20 that of LK (Fig. 6). The pattern of K$^+$ response with various Na$^+$ concentrations indicated an effect of Na$^+$ not only on K$^+$ activation, but also on K$^+$ inhibition, both types of response being clearly evident in HK membranes assayed with low ATP concentration.

Although low ATP concentrations are helpful for examining certain distinct effects of Na$^+$ and K$^+$, the necessity of using fragmented cell membranes to study the ATPase system does not allow the separate analysis of the effects of Na$^+$ and of K$^+$ on each side of the membrane. It is not possible to determine whether HK and LK membranes are different with respect to the interaction of one or both of the ionic species with one or both sides of the membranes. In studies of the pump with intact cells, Hoffman and Tosteson observed differences in both the affinity of the pump for extracellular K$^+$ and for intracellular Na$^+$ or K$^+$, or both (25). On the basis of their studies, they concluded that their data were compatible with a model consisting of rapidly equilibrating pump-ion complexes. They proposed that the rate of an actual transport step would be governed by the “fraction of pump sites which are loaded with appropriate ions on both the cis and trans surfaces.” In the present scheme, differences in affinities for Na$^+$ or K$^+$, or both, between HK and LK membranes may be associated with a difference in the steady state equilibrium between components of the Na$^+$-ATPase system, i.e. $E_0P \rightarrow E_0P$ and $E_1 \rightarrow E_0$. The former step would probably be associated with K$^+$ activation due to K$^+$-activated hydrolysis of $E_0P$; the latter step, with K$^+$ inhibition, would be due to interaction of K$^+$ with $E_0$. Evidence to support these effects of K$^+$ have been obtained recently by Siegel and Goodwin (26). A difference in the equilibrium of components would be a consequence of a difference in rate of a single step and would be reflected also in a quantitative difference in over-all Na$^+$-ATPase activity, as described previously (10).

Alternatively, the allosteric nature of Na$^+$-ATPase suggested...
in a number of studies in various laboratories (27-30) may explain the change in the properties of the system as the ATP concentration increases. A model in which Na\(^+\)-ATPase consists of subunits whose affinities for ligands are a function of the percentage of interacting subunits (lower scheme, Fig. 8) could explain the qualitative differences associated with the quantitative difference between HK and LK Na\(^+\)-ATPase activities.

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