Characterization of Adenosine Triphosphatase in Erythrocyte Membranes of the Cow[1] (36389)

K. S. KEETON AND J. J. KANEKO

Department of Clinical Pathology, School of Veterinary Medicine, University of California, Davis, California 95616

Intimately involved in the active transport of cations across erythrocyte membranes is a sodium–potassium-activated adenosine triphosphatase (Na–K–ATPase) (1, 2). ATPase in the membrane of red blood cells in humans is partitioned into two activity increments, one that is Na^+–K^+-dependent and one that is Na^+–K^+-independent, but both having an absolute requirement for magnesium (2). In the absence of Mg^{2+}, ATP is not hydrolyzed. The Na^+–K^+-dependent component is unaffected by either sodium or potassium added separately, but is greatly stimulated by the addition of Na^+ and K^+ together. Cardiac glycosides, such as ouabain, inhibit the Na^+–K^+-dependent increment but have no effect on the Na^+–K^+-independent increment (1, 2). These are general characteristics of an ATPase system for transport of Na^+ and K^+.

In various species, the membrane Na–K-ATPase activity and the intracellular cation concentration of the red blood cells are related. Dog and cat erythrocytes lack a membrane Na–K-ATPase (3, 4). These same cells contain low K^+ (8–10 mmoles/liter of rbc) and high Na^+ (100–110 mmoles/liter of rbc) concentrations with only a small concentration gradient existing across the membrane for the cations. In contrast, the high K^+ (100 mmoles/liter of rbc) low Na^+ (10 mmoles/liter of rbc) erythrocytes of man, rat, and rabbit have large concentration gradients across the membrane for Na^+ and K^+ and have substantial Na–K-ATPase activities (3, 5).

Erythrocyte cation concentrations in the cow are intermediate between those of man and the dog. Respective Na^+ and K^+ concentrations in the cow are approximately 70 and 20 mmoles/liter of rbc (6). The presence of a Na–K-ATPase in the membrane of cow erythrocytes has been both reported (7) and denied (8). This report presents evidence for the presence of an ATPase in the membrane of the red blood cell of the cow and describes its characteristics.

Materials and Methods. Membrane preparation. Venous blood was obtained aseptically from healthy Holstein Friesian cows, with heparin used as anticoagulant. Hemoglobin-free membranes for ATPase were prepared by a modification of the method of Burger, Fujii, and Hanahan (9). Approximately 60 ml of heparinized blood were centrifuged for 10 min at 1100*g* and at 4° in a Sorvall RC-2B centrifuge using a SS-34 rotor. The plasma and buffy coat were then removed and the cells were washed three times in ice-cold 0.002 *M* Tris–HCl, 0.15 *M* NaCl, pH 7.4. Removal of buffy coat was completed by careful aspiration after each centrifugation.

The washed red blood cells were then pooled, hemolyzed in 30 vol of a hypotonic solution (0.005 *M* $MgCl_2$, 0.005 *M* Tris–HCl, pH 7.65), and centrifuged at 27,000*g* and 4° for 15 min. The supernatant was decanted, and the membranes were washed 6 to 8 times with ice-cold hemolyzing solution until the supernatants were almost colorless. The resulting fluffy membranes, white or light pink, were stored in an equal volume of fresh hemolyzing solution at 2° until used. All ATPase assays were performed on the same day that the membranes were prepared.

The dry weight of the membranes in the membrane suspension was determined in du-

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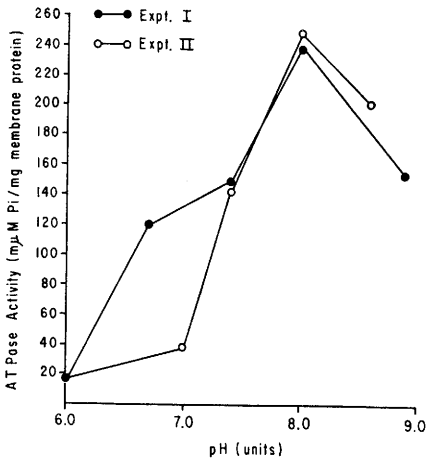

FIG. 1. Effect of pH on total ATPase activity. The conditions of the experiments were as follows: temperature, 37.5°; Tris–ATP, 2.5 m*M*; $MgCl_2$, 3.5 m*M*; NaCl, 150 m*M*; KCl, 5 m*M*; Tris–HCl, 25 m*M*; and time, 135 min.

plicate by drying 0.25-ml portions to constant weight at 140° in tared aluminum-foil cups and then computing the milligrams of dry membranes per milliliter of suspension. Protein concentration in the erythrocyte membrane was determined in quadruplicate by a colorimetric method (12).

Incubation and sampling. ATPase activity in the membrane of the red blood cell was measured by a modification of the method of Parker and Hoffman (10). The activity was determined at pH 8.0 and 37.5° in an assay system containing 1.0 ml of membranes, 0.25 ml of Tris–ATP, and various volumes of salt solutions, with Tris–HCl and ouabain added to a total volume of 2.5 ml. The salt solutions were prepared from the chloride salts of magnesium, sodium, and potassium. The final concentrations of the salts, Tris–ATP, Tris–HCl, and ouabain are given with the appropriate figures. The tubes were incubated in triplicate with gentle shaking for various periods between 90 and 135 min.

The reaction was begun by adding the substrate, ATP, to the incubation mixture. Samples of 0.5 ml were taken immediately after addition of the ATP and again at the end of the incubation period. Each sample was immediately mixed with 2 ml of ice-cold 10% trichloroacetic acid. After centrifugation, the resulting clear supernatants were analyzed in duplicate for inorganic phosphate (11). The difference in concentration of inorganic phosphate between the zero-time sample and the terminal sample was used to calculate the amount of orthophosphate (P_i) released. Activity was expressed as either nanomoles of P_i/mg of dry membranes or nanomoles of P_i/mg of membrane protein.

Results. When the red cell membranes were incubated at various pH values total ATPase activity rose sharply to a peak at pH 8.0 (Fig. 1), indicating a pH optimum in that vicinity.

Table I shows the effects of Mg^{2+}, Na^+, and K^+ on ATPase activity. In the absence of ions, ATP was not hydrolyzed. Sodium or potassium alone had no effect on activity, and a combination of sodium and potassium without magnesium produced only slight activation. A dependency of this ATPase on Mg^{2+} was indicated by the marked rise in activity in the presence of Mg^{2+} alone and the lack of activity in its absence. ATPase activity rose significantly above that in the presence of Mg^{2+} alone when Na^+ and K^+ were added in combination with the Mg^{2+}, and an apparent peak was reached at a Na^+:K^+ concentration ratio of 30:1 (Fig. 2). Na^+ alone or K^+ alone added to the Mg^{2+} gave an activity only slightly higher than that with Mg^{2+} alone. The activity level of

TABLE I. Effects of Magnesium, Sodium, and Potassium on ATPase Activity of Red Cell Membranes.[a]

Ionic composition of the incubation media (m*M*)	ATPase activity (nmoles of P_i released/mg) of: Dry membranes	Membrane protein
No ions present	0.0	0.0
Mg^{2+}, 2.5	29.8	98.7
Na^+, 150	0.0	0.0
K^+, 150	0.0	0.0
Na^+, 150 + K, 5	6.8	22.3

[a] The reaction mixtures contained the following: Tris–ATP, 2.5 m*M*; Tris–HCl, 100 m*M*; and the various ions in concentrations as shown. Incubation was carried out at 37.5° and pH 8.0 for 120 min.

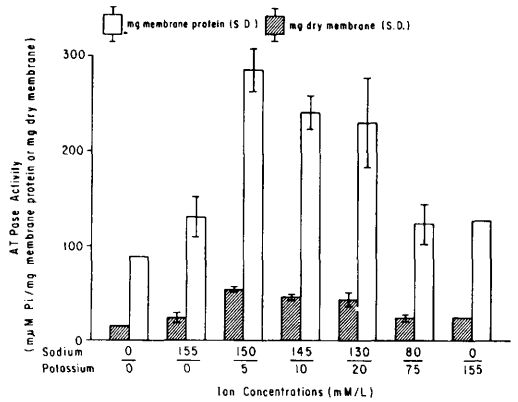

FIG. 2. Effect of varying sodium and potassium ion concentrations on the magnesium-dependent ATPase activity. The conditions of the experiments were as follows: temp, 37.5°; pH 8.0; Tris–ATP, 2 mM; $MgCl_2$, 4.5 mM; Tris HCl, 25 mM, and time, 135 min. The results represent the average of two experiments with the standard deviation given at each ionic concentration except for the concentrations of 0:155 sodium and potassium and 0:0 sodium and potassium. To keep tonicity the same, the total concentration of sodium plus potassium was maintained at $\cong$155 mM in all tubes except that of 0:0.

the 150:5 concentration was elevated significantly over the 155:0 ($p < 0.02$). Thus, the markedly stimulated ATPase activity indicated a Na^+–K^+-dependent ATPase activity in addition to the Mg^{2+}-dependent activity (Table II).

Table II shows the effect of ouabain and the activities produced with the various ion concentrations. Ouabain had no effect on the Mg–ATPase increment but inhibited the Na–K–ATPase increment. This inhibition was maximal at ouabain concentrations between 10^{-3} to 10^{-5} M (Fig. 3). At maximal inhibition, the reduction of the total ATPase activity indicated that the Na–K–ATPase increment was approximately 50% of the total. Also of note was the sigmoid shape of the activity curve.

As shown (Fig. 4), the total ATPase and ouabain-insensitive (Mg–ATPase) activities peaked at a Mg^{2+} concentration of approximately 2.5 mM. The ouabain-sensitive Na–K–ATPase increment however, peaked at approximately 1.5 mM Mg^{2+}. The activity peaks observed for the total ATPase and for ouabain-sensitive activities (Fig. 4) corresponded to Mg^{2+}:ATP respective concentration ratios of 1:1 and 0.6:1. A greater sensitivity to a deviation from the 1:1 Mg^{2+}:ATP concentration ratio was indicated by the more pronounced decrease of the ouabain-sensitive increment relative to the ouabain-insensitive increment.

The Michaelis constant of ATPase for ATP (K_m ATP) was derived by the Lineweaver-Burk formulation (Fig. 5). The line of regression was calculated by the least squares method. The linear relation between 1/ATP and 1/activity was statistically significant ($r = 0.92$, $p < 0.001$). As estimated from the slope and the V intercept, the K_m ATP was 2.1×10^{-4} M.

Discussion. The ATPase activities of erythrocyte membranes in the cow measured under various conditions revealed two ATPase increments, both requiring magnesium for activity. The first increment, Mg–ATPase, did not require sodium and potassium for stimulation and was insensitive to ouabain. The second increment required sodium and potassium for stimulation and was completely inhibited by ouabain. This ouabain-sensitive increment made up approxi-

TABLE II. Effects of Magnesium, Sodium, and Potassium on ATPase Activity With and Without Ouabain Present.[a]

Ionic composition of incubation media	ATPase activity (nmoles P_i released/mg) of: Dry membranes	Membrane protein
Mg^{2+}	44.0	205.9
Mg^{2+} + ouabain	37.2	174.0
Na^+	46.4	217.6
Na^+ + ouabain	32.3	151.2
K^+	22.6	105.9
K^+ + ouabain	37.5	175.8
$Na^+ + K^+$	80.0	374.4
$Na^+ + K^+$ + ouabain	42.4	197.5

[a] The experimental conditions were as follows: temp, 37.5°; pH 8.0; Tris–ATP, 2.5 mM; $MgCl_2$, 5 mM; Tris–HCl, 25 mM; and time, 135 min. Sodium chloride, potassium chloride, and ouabain, when present, were at respective concentrations of 170 mM, 6 mM, and 1×10^{-4} M.

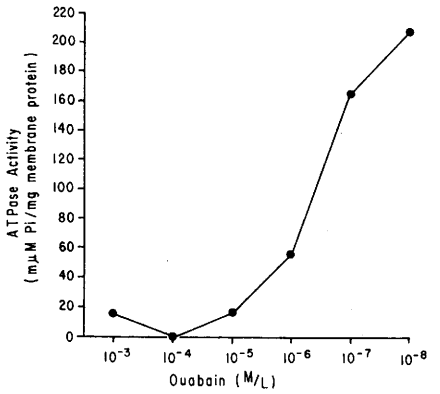

FIG. 3. Effect of ouabain on the Na–K–Mg–ATPase activity. The following experimental conditions were employed: temp, 37.5°; pH 8.0; Tris–ATP, 2.5 mM; $MgCl_2$, 5 mM; NaCl, 145 mM; KCl, 5 mM; Tris–HCl, 25 mM; and time, 135 min. Only the activity of the Na–K-dependent component is shown.

mately 50% of the total ATP hydrolyzing activity.

The pH and Mg^{2+}:ATP concentration ratio required for maximum activity, and also the K_m ATP value for the enzyme, were similar to values reported for man (2, 13, 14).

The optimal pH value of 8.0 found for the cow enzyme is the same as found (13) for the human erythrocytic ouabain-sensitive ATPase activity. The 1:1 concentration ratio required for Mg^{2+} and ATP suggests that a Mg^{2+}–ATP complex is the actual substrate of the enzyme. In equimolar amounts, the very high affinity constant between Mg^{2+} and ATP would allow very little magnesium to exist in the free state (15). Since the ouabain-sensitive and ouabain-insensitive activity increments both decreased as the Mg^{2+} concentration was increased, the free Mg^{2+} might have interfered by competing with the Mg^{2+}–ATP complex for the enzyme or might have altered the enzyme by binding at another site independent of the active substrate site.

The K_m ATP value of 2.1×10^{-4} M for the cow enzyme is close to the 2.85×10^{-4} M value found for the human red cell stromal ATPase (14). Red cell ATP concentrations reported for Holstein cows (16) and man, (17) when expressed per liter of cell water, are approximately 10 times the K_m value in both species. However, since ATP may be transferred to the ATPase within the microenvironment of the membrane (18), the intracellular concentration of ATP might not play a direct role in regulating the enzyme activity.

The marked stimulation by sodium and potassium of ATPase activity in red cells of the cow was dependent on the combined presence of both the ions and on their relative concentrations. The observation of an optimal Na^+:K^+ ratio is consistent with the demonstration of sodium-binding sites and potassium-binding sites in membranes of red cells in humans (19). The asymmetrical nature of cation stimulation of the red cell ATPase requires sodium ions on the inside and potassium ions on the outside of the cell membrane (19). Significant deviation from the optimal 30:1 Na^+:K^+ ratio probably resulted in competition of Na^+ or K^+ for sites normally occupied by the alternate ion with an accompanying decrease in activity. Although the requirement of an optimal Na^+:K^+ ratio for maximal activation was demonstrated for the bovine membrane

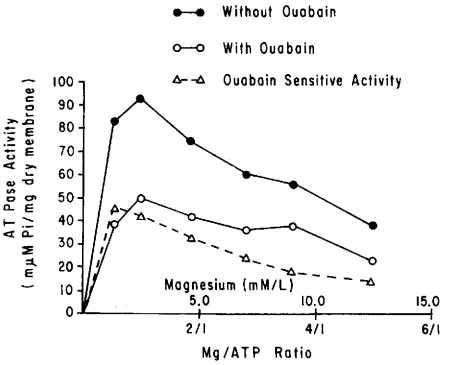

FIG. 4. Effect of magnesium on total ATPase activity, ouabain-sensitive ATPase activity, and ouabain-insensitive ATPase activity. The following conditions were employed: temp, 37.5°; pH 8.0; Tris–ATP, 2.5 mM; NaCl, 145 mM; KCl, 5 mM; and time, 120 min. The activity without ouabain present (●) represents the total ATPase activity. The activity with ouabain present at 2×10^{-4} M (○) represents the ouabain-insensitive activity. The difference between these two curves represents the ouabain-sensitive activity component (△- -).

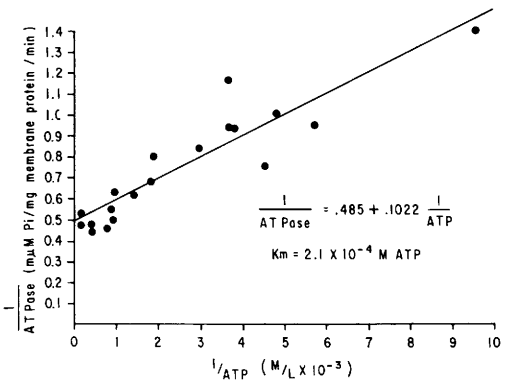

FIG. 5. Lineweaver-Burk plot for K_m ATP of total ATPase activity. The experimental conditions were as follows: pH 8.0; temp, 37.5°; equimolar amounts of Tris–ATP and $MgCl_2$ from 1.05×10^{-4} to 6×10^{-3} *M*; NaCl, 150 m*M*; KCl, 5 m*M*; Tris–HCl, 125 m*M*; and time, 75 min. The points shown represent three separate experiments using membranes derived from three cows.

ATPase, the asymmetric stimulation by these two cations can only be assumed to exist since the membrane preparation used in these experiments did not permit variation of ion concentrations on each side of the membrane.

Inhibition by ouabain of the synergic stimulation of the bovine ATPase by Na^+ and K^+ was consistent with the findings for erythrocyte ATPase derived from humans (2, 20) and other species (5, 21, 22). This inhibition of the Na–K–ATPase system by the cardiac glycosides, because of its constancy, has been described as one of the characteristics that defines this particular enzyme system. The sigmoid slope of the curve relating enzymatic activity and ouabain concentration indicated a possible allosteric inhibition of the enzyme by the glycoside. This assumption is supported by evidence (23) that the glycosides must be bound to a site on the cell membrane which is different from the transport site with possible membrane conformational changes in the area of the Na–K–ATPase resulting in its inhibition.

Confirmation of the presence of a Na–K–ATPase and Mg–ATPase in the membrane of the low K^+ red cell in the cow indicates that this cell is qualitatively similar to that in man and other species with red cells possessing high K^+ and low Na^+ concentrations. The characteristics found for the membrane ATPase in the red cell of the cow indicate that the cow enzyme parallels very closely the properties of an enzyme intimately involved in the active transport of electrolytes across biological membranes and would mean that the red cell of the cow, like that in other species, maintains its cation composition and volume by the action of a K^+–Na^+ exchange pump.

Summary. The cow red cell membrane was demonstrated to possess a Mg-dependent adenosine triphosphatase (Mg–ATPase) and a Na–K-dependent ATPase (Na–K–ATPase), the activity of which was completely abolished by the presence of ouabain. The pH optimum, the optimum Mg^{2+}:ATP concentration ratio, the K_m ATP for the enzyme, and the ouabain concentration required for inhibition of activity were similar to those of the membrane ATPase of human erythrocytes. The cow enzyme was also sensitive to the medium Na^+:K^+ concentration ratio. The cow red cell membrane, like that of other species, was confirmed to possess an enzyme system with the characteristics of a transport ATPase.

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