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ACTIVE POTASSIUM TRANSPORT IN RETICULOCYTES OF HIGH-K^+ AND LOW-K^+ SHEEP

PHILIP B. DUNHAM and RHODA BLOSTEIN

Department of Biology, Syracuse University, Syracuse, N. Y. (U.S.A.) and Division of Hematology, Royal Victoria Hospital, Montreal, Quebec (Canada)

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SUMMARY

The kinetics of active K^+ transport were studied in immature red blood cells cells from high-K^+ and low-K^+ sheep, particularly with respect to the effects of varying intracellular K^+ concentration, $[K]_i$. Comparison was made with active transport, or pump, activity in mature high-K^+ and low-K^+ red cells. Reticulocytes from both types of sheep had much higher maximal active K^+ influxes than did mature cells. In both types of reticulocytes, and in mature high-K^+ cells as well, the pump was relatively insensitive to increasing $[K]_i$. In contrast, intracellular K^+ markedly inhibited the pump in mature low-K^+ cells. Active K^+ transport in low-K^+ reticulocytes, however, as in mature low-K^+ cells, is stimulated by specific isoimmune anti-L serum. Therefore the K^+ pumps of high-K^+ and low-K^+ reticulocytes have similar kinetic properties. Maturation of the red cells, involving inactivation of most of the pump activity in both cell types, results in mature high-K^+ and low-K^+ cells with K^+ pumps of very different kinetic characteristics.

INTRODUCTION

Sheep are dimorphic with respect to the ability of their red blood cells to pump Na^+ and K^+. High potassium sheep have red cells with the typical high K^+ and low Na^+ concentrations, while low potassium sheep have red cells with low K^+ and high Na^+ concentrations [1, 2]. The difference in cation composition is correlated with higher pump fluxes and lower passive fluxes of Na^+ and K^+ in high-K^+ cells as compared to low-K^+ cells [3]. This genetically determined dimorphism [4, 5] may be due in part to a difference in number of active transport sites per cell [6]. In addition the pump sites on high-K^+ and low-K^+ cells have different kinetic properties. The Na^+-K^+ pump in low-K^+ cells is much more sensitive to inhibition by intracellular K^+ than in high-K^+ cells [7]. This kinetic difference is also indicated by a greater inhibition of Na^+-ATPase activity by K^+ in membranes of low-K^+ cells and in a different response to oligomycin [8, 9]. Finally pumps in high-K^+ cells appear to have a higher affinity for ouabain [6, 10].

The M-L blood group antigen system in sheep is associated with the high-

low-K^+ dimorphism in that nigh-K^+ cells have only M antigen and the L antigen is found only on low-K^+ cells [11]. Anti-L antibody, raised by injecting low-K^+ cells into a high-K^+ sheep, stimulates active K^+ transport in low-K^+ cells 4-fold or more [12]. The pumps of anti-L treated cells have reduced sensitivity to inhibition by intracellular K^+ [13, 14]. Although early work provided evidence for an increased number of ouabain sites on anti-L treated cells [13], a more recent study has not confirmed this finding [10]. Anti-L treatment also reduces passive K^+ transport in low-K^+ sheep cells [15, 16].

In low-K^+ sheep, reticulocytes have a cation composition like high-K^+ cells [17, 18] and have a much higher pump flux than mature low-K^+ cells [19, 20]. The pump rate and K^+ concentration decline as the cells mature during several weeks in circulation. There are also preliminary results indicating that low-K^+ reticulocytes have more ouabain binding sites than mature low-K^+ cells [21]. There is no information on these characteristics of reticulocytes from high-K^+ sheep.

Blostein et al. [22] compared the kinetics of Na^+, K^+-activated ATPase in reticulocytes and mature red cells from high-K^+ and low-K^+ sheep. The procedure involved measuring Na^+-dependent ATPase activity at 0.2 μM ATP and examining the effect of K^+ and oligomycin on the Na^+-ATPase. The Na^+-ATPase activities in both high- and low-K^+ reticulocytes were much higher than in mature cells and the kinetics of the Na^+-ATPase were much more similar to one another in the two types of reticulocytes than they were in mature high- and low-K^+ cells. In fact, in one respect (oligomycin inhibition) reticulocytes, like anti-L treated low-K^+ cells, resembled mature high-K^+ cells, in another respect (inhibition by K^+), they resembled mature low-K^+ cells, although in low-K^+ cells the inhibition was less marked in reticulocytes than in mature cells.

The question then is whether, and to what extent, these properties of Na^+, K^+-ATPase reflect the kinetics of the cation pump. In this paper we describe a study of the kinetics of active K^+ transport in reticulocytes from high- and low-K^+ sheep, particularly with regard to the effect of varying intracellular K^+ concentrations. A preliminary report of these results has been published [23].

METHODS

Active erythropoiesis was stimulated in adult sheep by bleeding on three successive days (1 liter per day). Reticulocyte-rich fractions of red cells were obtained by gradient centrifugation from blood drawn six days after the end of the bleeding. After the initial bleeding the sheep could be maintained chronically anemic ($\approx 5\,\%$ reticulocytes) by bleeding approximately weekly (800 ml). A total of 3 high- and 3 low-K^+ sheep were used in the experiments.

Fractions containing up to 80 % reticulocytes were obtained from the top fraction of a 20–30 % Dextran gradient after centrifugation at 12 000 $\times$ g for 30 min [24]. Reticulocyte counts were made on smears stained with brilliant cresyl blue [25]. Sufficient cells were counted so as to give standard errors $\leqq 5\,\%$.

The cation composition of the cells was varied using the method of reversibly increasing cation permeability with nystatin [26] modified for use with sheep red cells (Gunn, R. B., personal communication). The cells were exposed to nystatin in solutions containing: 0–50 mM KCl, 135–85 mM NaCl, (KCl+NaCl = 135 mM);

68 mM sucrose, 10 mM Tris · Cl, pH 7.5; and 50 μg/ml nystatin (Mycostatin, G. R. Squibb Sons, Inc.). Stock solutions of nystatin were made the day before use at 5 mg/ml in methanol. The cells were exposed to the solutions containing nystatin at hematocrits of 5 % or less in an ice bath for 40 min. The cells were then washed free of nystatin by six successive centrifugations in the cold in solutions of composition identical to the incubating solutions, except without nystatin. The cells were then washed twice in a solution containing 150 mM NaCl, 5 mM glucose and 10 mM Tris · Cl, pH 7.5. Samples of suspensions were then set aside for determination of cell volume relative to fresh cell volume (calculated from the hematocrit of the suspension and the hemoglobin concentration of a hemolysate of the suspension). The above procedure for altering cellular Na^+ and K^+ concentrations results in negligible hemolysis. Cell volumes and the active and passive fluxes of K^+ are the same as in fresh cells of the same cation composition.

Unidirectional K^+ influxes were measured using $^{42}K^+$. Aliquots of 0.02–0.05 ml of packed cells were suspended in 0.1 ml of a solution containing 150 mM NaCl, 5 mM glucose and 10 mM Tris · Cl, pH 7.5, and 10^{-3} M ouabain as desired. The remainder of the procedure for measuring and calculating the fluxes was as described previously [27]. The tubes containing the cell suspensions were incubated at 37 °C for 10–15 min. The fluxes were then started by adding to each tube 1 ml of a solution identical to that in which the cells were suspended except that part of the Na^+ was replaced with K^+ containing $^{42}K^+$. The cells were exposed to $^{42}K^+$ for 30 min. External K^+ concentration, $[K]_0$, was 3–5 mM. Active K^+ influx was taken as the ouabain-sensitive flux. Intracellular K^+ concentrations, $[K]_i$, were also measured as described in the earlier report. During the fluxes (incubation for 30 min) significant changes in $[K]_i$ occurred in reticulocytes, to a maximum of about 3 mmol/l cells. The $[K]_i$ values presented are averages for the 30 min incubation, i.e. the mean of the $[K]_i$ in cells after incubation in the absence of ouabain (in which the net influx occurred) and the $[K]_i$ in cells after incubation in ouabain (net influx inhibited).

In some experiments cells were exposed to anti-L antiserum before measuring the K^+ influx. Antiserum against the L-antigen was raised by intramuscular injection into a high-K^+ sheep of washed low-K^+ cells in Freund's complete adjuvant [28]. Before use the serum was heated at 56 °C for 20 min to inactivate complement and dialyzed for 30 h at 4 °C against 50 volumes of a solution containing 150 mM NaCl, 5 mM glucose, and 10 mM Tris · Cl, pH 7.4. After nystatin-treatment, aliquots of cells were exposed to serum at 5–10 % hematocrit for 30 min at 32 °C. The cells were then washed 3 times by centrifugation before measurement of K^+ influxes.

The methods for obtaining membrane preparations and for measuring Na^+-ATPase activity were as described previously [8, 9].

In all figures, the points represent means of two determinations, and the curves were fitted by eye.

RESULTS

Fig. 1 shows active K^+ influx in mature high- and low-K^+ cells of non-anemic sheep. (The scales on the ordinates are different for high- and low-K^+ cells.) In these

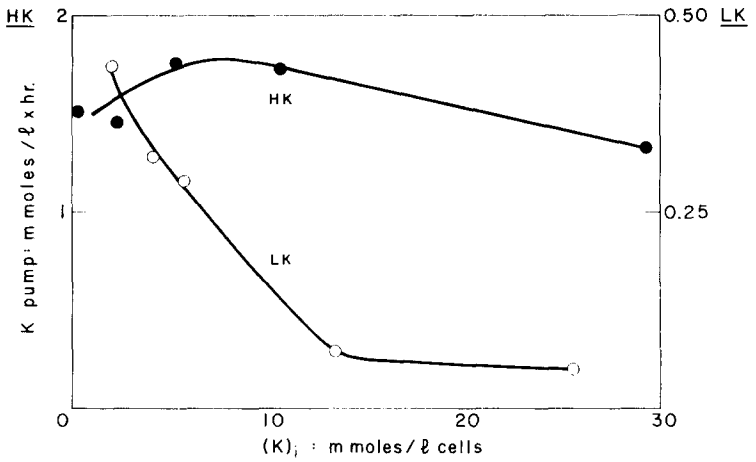

Fig. 1. Active K^+ influx (K^+ pump: mmol/l cells per h) in red cells from nonanemic high-K^+ (HK) and low-K^+ (LK) sheep. The ordinates for high- and low-K^+ cells are on different scales. These results serve as controls for those in subsequent figures in that these preparations contaned less than 1 % reticulocytes. The intracellular K^+ concentrations, $[K]_i$, in this experiment and in those shown in subsequent figures, were altered using nystatin as described in the text. The results are representative of 8 similar experiments.

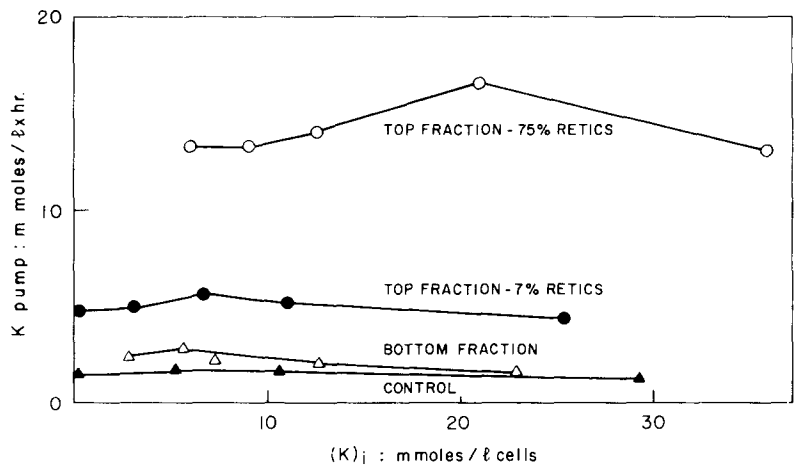

Fig. 2. Active K^+ influx in red cells from anemic high-K^+ sheep. The lowest curve (control) is taken from Fig. 1. Top and bottom fractions were taken from a Dextran gradient, as described in the text. The results for the top fraction with 7 % reticulocytes and bottom fraction were obtained with cells from the same sheep on the same day.

preparations reticulocyte counts were less than 1 %. The pump fluxes were measured in cells with various $[K]_i$ values. As shown previously [7], the K^+ pump in low-K^+ cells was strongly inhibited as $[K]_i$ was increased (90 % inhibition as $[K]_i$ is raised from 2 to 26 mmol/l cells). In high-K^+ cells $[K]_i$ was only slightly inhibitory (15 % inhibition as $[K]_i$ is raised from 5 to 29 mmol/l cells). In addition there was a slight stimulation of the K^+ pump (9 %) in high-K^+ cells as $[K]_i$ was increased from 1 to 5

mmol/l cells. Stimulation of the K^+ pump by increasing $[K]_i$ was first observed in high-K^+ goat red cells [15, 27]. A similar phenomenon has been described in human red cells [29]. The three effects of $[K]_i$ on the K^+ pump on sheep red cells (marked inhibition in low-K^+ cells; slight stimulation and inhibition in high-K^+ cells) were highly reproducible in the present study.

Fig. 2 shows the results of similar experiments on cells from anemic high-K^+ sheep. The bottom curve (control), for mature cells from a non-anemic sheep, was taken from Fig. 1. In a reticulocyte-rich fraction (top curve) maximal pump activity was about 10-fold higher than in mature cells. Also shown in Fig. 2 are data for a fraction containing young cells (top fraction from a Dextran gradient) but with a lower number of reticulocytes, and for mature cells from an anemic sheep, i.e. the bottom fraction of a Dextran gradient. In reticulocytes from high-K^+ sheep, as in mature high-K^+ cells, changing $[K^+]$ had little effect on the K^+ pump, with indications of slight stimulation as $[K]_i$ was raised from a low concentration, and slight inhibition as $[K]_i$ was increased further. The stimulation and inhibition in the reticulocyte-rich preparation in Fig. 2 were both of the order of 22 %.

Fig. 3 shows K^+ pump fluxes from anemic low-K^+ sheep. The curve for mature cells (control) was taken from Fig. 1. As reported previously [19, 20], the pump flux in low-K^+ reticulocytes was much higher than mature low-K^+ cells, some 20-fold higher in the present work. Indeed the pump flux is higher in low-K^+ reticulocytes than in mature high-K^+ cells.

A striking difference was observed in the kinetics of the K^+ pump between mature low-K^+ cells and low-K^+ reticulocytes. Varying $[K]_i$ between less than 1 and 38 mmol/l in preparations of young low-K^+ cells had little effect on the K^+ pump (maximum possible inhibition, 12 %) whereas the same change in $[K]_i$ in mature

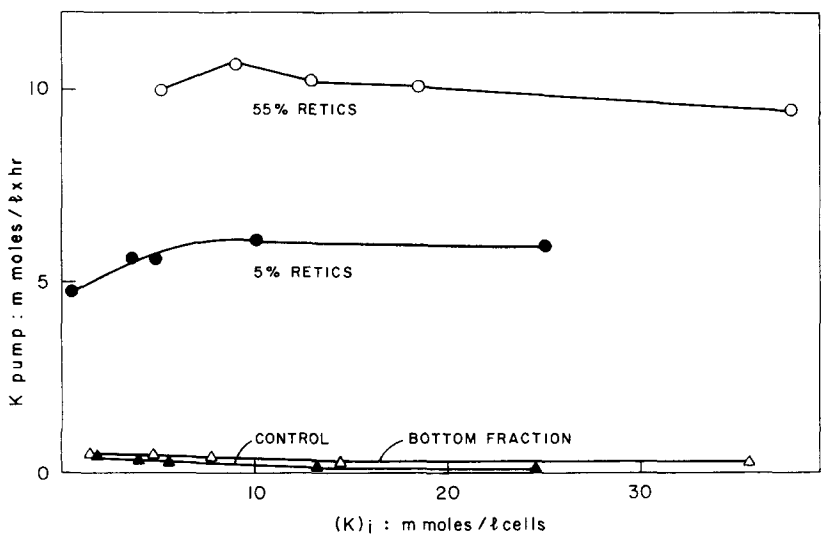

Fig. 3. Active K^+ influx in red cells from anemic low-K^+ sheep. The lowest curve (control) is taken from fig. 1. The results for the 55 % reticulocyte preparation and the bottom fraction were obtained with cells from the same sheep on the same day.

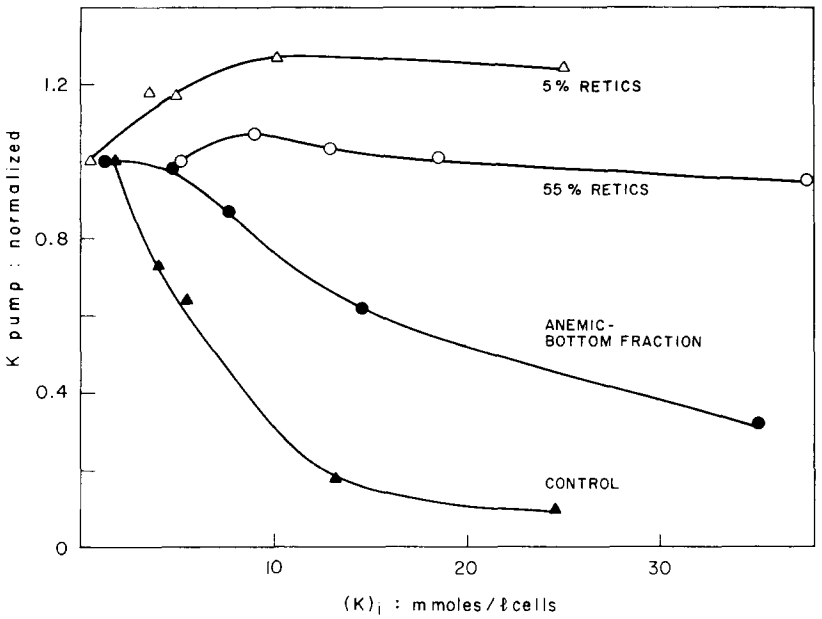

Fig. 4. Results from Fig. 3 normalized by setting the K^+ pump flux at the lowest $[K]_i$ equal to 1.0.

low-K^+ cells inhibited the pump by 90 % (Fig. 1). In addition there was an indication of stimulation of the pump in young low-K^+ cells as $[K]_i$ was raised from near zero to 10 mol/l (29 % stimulation in the 5 % reticulocyte preparation, Fig. 3). Thus in their kinetic response to varying $[K]_i$, young low-K^+ cells are very much like young and mature high-K^+ cells (Figs. 1 and 2) and are very different from mature low-K^+ cells (Fig. 1).

The differences between low-K^+ reticulocytes and mature low-K^+ cells is shown to better advantage in Fig. 4, in which the data in Fig. 3 have been normalized by setting the pump flux at the lowest $[K]_i$ equal to 1.0. In young cells there is stimulation at low $[K]_i$ and slight, if any, inhibition at higher concentrations. The apparent difference between the curves for the two reticulocyte preparations is probably not real, but due to the fact that the two curves were normalized to different low $[K]_i$ values. The apparent action of $[K]_i$ is not likely due to varying $[Na]_i$. Although $[Na]_i$ and $[K]_i$ were varied reciprocally, $[Na]_i$ was decreased by about 10 % as $[K]_i$ was raised from near zero to 10 mmol/l. The minimum $[Na]_i$ in any preparation of cells was 75 mmol/cells.

Passive K^+ influxes in mature cells were similar to those reported previously: about 0.2 and 0.05 mmol/l cells per h in high- and low-K^+ cells, respectively, at 5 mM $[K]_i$. The passive K^+ fluxes were much higher in both types of reticulocytes: 1.1 to 3.3 mmol/l per h in low-K^+ cells and 1.0 to 2.3 mmol/l per h in high-K^+ reticulocytes. The passive fluxes varied more in reticulocytes than in mature cells, and were not correlated with the pump fluxes. No attempt was made to determine the source of this variability.

Experiments using the anti-L antibody indicate a stimulation of active transport in low-K^+ reticulocytes by pretreatment with the antibody. (There was an earlier

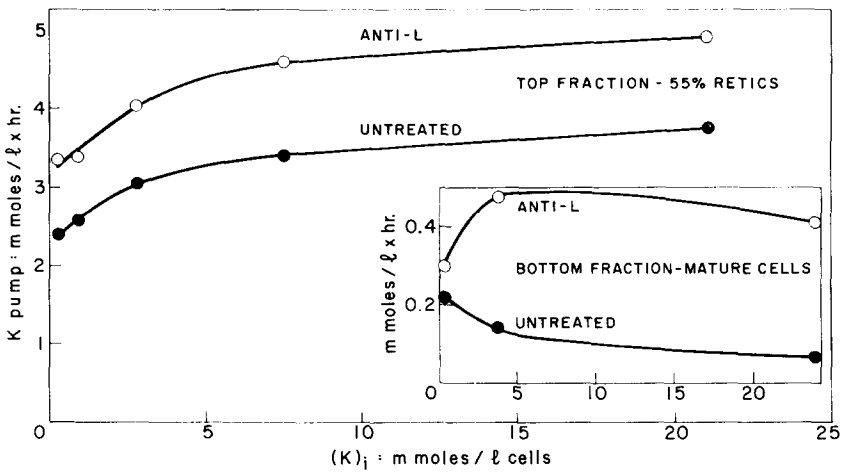

Fig. 5. Active K^+ influx in anti-L-treated cells from an anemic low-K^+ sheep. Cells from top and bottom fractions (all obtained from the same sheep on the same day) were divided into two aliquots after nystatin-treatment. One aliquot was treated with anti-L antiserum as described in the text. Results for the top and bottom fractions appear in the main part of the figure and in the inset, respectively.

report of little or no effect of anti-L on the K^+ pump in these cells [20]). Fig. 5 shows the effect on the K^+ pump of treating with anti-L both a reticulocyte-rich preparation of low-K^+ cells and also mature low-K^+ cells from the same sheep taken at the same time from the bottom fraction of the Dextran gradient. Stimulation of the K^+ pump by anti-L was observed in both preparations of cells at all $[K]_i$ values. The relative stimulation was greater in the bottom fraction (nearly 5-fold at the highest $[K]_i$). However, the absolute stimulation is of the order of 1.0 mmol/l per h in the reticulocytes and 5-fold lower, 0.2 mmol/l per h, in the mature cells. In another similar experiment the stimulation in low-K^+ reticulocytes was 1.6 mmol/l per h.

In the cells from the bottom fraction, anti-L appeared to alter the kinetic properties of the pumps in addition to stimulating their maximum velocity. The untreated cells from the bottom fraction showed a marked inhibition of the pump by K_i (65 % inhibition, $[K]_i$ increased from near 0 to 24 mmol/l), similar to that in mature low-K^+ cells in Fig. 1. However, the pump of the anti-L-treated cells of the bottom fraction was only slightly inhibited by K_i (13 % inhibition, $[K]_i$ increased from 3 to 24 mmol/l); there was an indication of stimulation (56 %) at low $[K]_i$. Similar qualitative changes in the shape of the curve relating the K^+ pump flux to $[K]_i$ after treatment with anti-L have been observed previously [13–15, 27] and have been attributed to a change in the relative affinities for Na^+ and K^+ at the intracellular aspects of the pump sites, perhaps a reduction in the affinity for K^+ as an inhibitor [27].

There was no indication of a change in the shape of the curve relating the K^+ pump flux to $[K]_i$ in low-K^+ reticulocytes after treatment with anti-L (Fig. 5). The relative stimulatory effects of anti-L were about the same at the lowest $[K]_i$, at which K_i should have a negligible inhibitory effect, and at a $[K]_i$ of 20 mmol/l, at which concentration a change in inhibitory effect of K_i brought about by anti-L should be

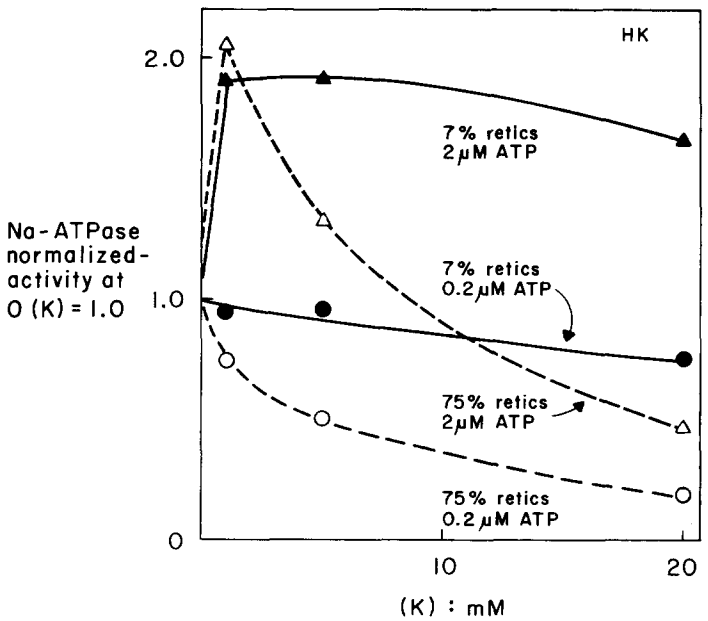

Fig. 6. Na^+-ATPase activity in two preparations of membranes from red cells from anemic high-K^+ sheep. Enzyme activities were measured as a function of [K] at two ATP concentrations. Activities were normalized by setting the activity in K^+-free medium equal to 1.0.

apparent. The stimulatory effects at the low and high $[K]_i$ values were 37 and 32 %, respectively. Therefore the stimulation of the K^+ pump by anti-L in low-K^+ reticulocytes can be attributed entirely to an effect on maximum velocity of the pump.

Anti-L antiserum has been shown to reduce passive K^+ influx in low-K^+ sheep red cells [15, 16, 30]. However, anti-L treatment has been shown not to reduce passive K^+ influx in low-K^+ reticulocytes [30].

Qualitatively the effects of K_i on the pump are similar in reticulocytes and mature cells from high-K^+ sheep as shown in Fig. 2. In contrast Na^+-ATPase measured at 0.2 μM ATP in mature high-K^+ cells is not inhibited by K^+ whereas the enzyme from high-K^+ reticulocytes is [22]. Fig. 6 shows the results of experiments in which Na^+-ATPase activity was measured in 2 preparations of membranes from high-K^+ reticulocytes, at various K^+ concentrations, and at ATP concentrations both 0.2 μM as used in the previous study [22] and at a higher ATP (2.0 μM). The results indicate that the inhibitory action of K^+ is considerably reduced as the ATP concentration is increased. This mutual interaction of ATP and K^+, though well documented for (Na^+, K^+)-ATPase from a variety of sources, including mature high-K^+ Na^+-ATPase , is not observed with mature low-K^+ sheep cells (Fig. 5 of ref. 9). Thus the discrepancy between kinetics of K^+ inhibition of the pump and of Na^+-ATPase in high-K^+ reticulocytes is evident to varying extents depending on the ATP concentration.

DISCUSSION

Both high- and low-K^+ reticulocytes have much higher K^+ pump fluxes than the mature cells. The kinetic characteristics of the pump in the two types of reticulocytes are similar in that both are relatively insensitive to varying $[K]_i$. In terms of the characteristics of the Na^+-ATPase, high- and low K^+ reticulocytes are also similar to one another and furthermore are more similar to one another than are preparations of Na^+-ATPase from mature high- and low-K^+ cells [22].

Differentiation of red cells in both high- and low-K^+ sheep is accompanied by large reductions in total pump activity, on the order of 10-fold in high-K^+ cells and 20-fold in low-K^+ cells. At the same time there are changes in kinetic characteristics, and the changes proceed very differently in the two cell types. As low-K^+ cells mature their K^+ pumps become much more sensitive to inhibition by $[K]_i$; concomitantly, all indication of stimulation of the pump at low $[K]_i$ is lost. At the same time the Na^+-ATPase of low-K^+ reticulocytes becomes more sensitive to inhibition by K^+ and undergoes a high-K^+-like to a low-K^+-like transition with respect to the response to oligomycin [22].

As high-K^+ cells mature there is no striking change in the kinetic response of the K^+ pump in intact cells to varying $[K]_i$. The results on the kinetics of the K^+ pump and Na^+-ATPase, respectively, appear to contrast in that Na^+-ATPase from high-K^+ reticulocytes is more sensitive to K^+-inhibition than the enzyme from mature high-K^+ cells, but inhibition of the pump by K_i does not change with maturation. However, K^+-inhibition of Na^+-ATPase becomes much less apparent as ATP concentration is raised. If it were possible to measure pump activity at very low ATP concentrations, K^+ might inhibit more strongly in high-K^+ reticulocytes than in mature cells.

The results are consistent with the view that both high- and low-K^+ reticulocytes have higher numbers of pump sites than mature cells of the two cell types. It appears also that the pump sites are heterogeneous with respect to their kinetic properties and with different ranges of properties on the two cell types. Of relevance in this context is the varying degree to which the various kinetic properties resemble either high- or low-K^+ cells. If differentiation into normal mature red cells involves a partial or complete inactivation of most of the pump sites on both types of cells, then this change is selective with respect to the kinetic properties of the pumps. Thus mature cells are kinetically distinct not only from the precursor reticulocytes but from each other. Finally the genetic difference between high- and low-K^+ animals determines that the selective changes proceed differently in the two cell types, and from reticulocytes with similar properties, very different mature cells differentiate in high- and low-K^+ sheep.

The pump sites on low-K^+ reticulocytes like the pumps of mature low-K^+ cells, are responsive to anti-L, despite having kinetic characteristics different from the pumps on mature cells. The magnitude of the response is greater in immature than in mature low-K^+ cells, consistent with the indication that low-K^+ reticulocytes have many more pump sites than mature low-K^+ cells. Low-K^+ reticulocytes do have as much L-antigen as mature low-K^+ cells, as judged by their ability to absorb L-activity from antisera [20]. In contrast to the present work, Tucker and Ellory

[20] found little or no stimulation of the K^+ pump in low-K^+ reticulocytes by anti-L; the reason for the difference in results is not apparent.

The process of differentiation of erythrocytes in anemic sheep may involve replacement as well as actual changes in circulating cells. That differentiation is of major importance was evident in studies of Lee et al. [19], in which reticulocytes were labelled with ^{59}Fe. At intervals blood samples were fractionated by density gradient centrifugation, and the density distributions of ^{59}Fe, red cell $[K]_i$, and active K^+ transport were measured. The labelled cells remained in circulation throughout the course of the experiment, during which period the differentiation of their transport activity was demonstrated.

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