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(Na^+–K^+)-ACTIVATED ATPase IN CATTLE ERYTHROCYTES

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SUMMARY

1. In the presence of 150 mM Na^+, increasing K^+ levels initially stimulated ($K^+ < 5$ mM) and then inhibited ouabain-sensitive ATPase activity in cattle erythrocyte ghosts.

2. Ouabain-sensitive K^+ uptake measurements in high Na^+ medium showed that the external affinity for K^+ was about the same for high or low K^+ cells.

3. K^+ inhibition of ATPase was greatest in ghosts derived from low K^+ cells, and least in ghosts from high K^+ cells, but a spectrum of sensitivities was found, correlating with the original red cell K^+ level.

4. Sensitization with sheep anti-L reagent gave an increased overall ouabain-sensitive ATPase activity, with a small decrease in the apparent affinity for the K^+-inhibition component.

INTRODUCTION

Sheep and goats show a simple polymorphism in their red cell potassium levels, being either high (HK) or low (LK) potassium type [1, 2]. This difference has been shown to be under simple genetic control [3], and is primarily mediated by changes in the kinetic properties of the red cell sodium pump, particularly in the internal affinity of the pump for K^+ [4–6]. Potassium type has also been shown to be associated with the M-L antigen system [7], and LK sheep and goat red cells react specifically with the anti-L antibody giving an increased sodium pump activity. The mechanism of anti-L stimulation of the Na^+ pump is not completely resolved, although its principal effect seems to be to alter the internal K^+ affinity of LK type Na^+ pumps [5–8].

In contrast to sheep and goats, cattle show a continuous distribution with respect to red cell K^+ levels, from 15–110 mmoles/l cells [9–11]. Further, cells with up to 70 mmoles K^+/l cells, which would certainly be classified as HK type by analogy with sheep and goats, show an increased sodium pump activity following sensitization with anti-L [11]. Differences between red cell potassium levels in cattle red cells would probably be mediated by changes in either passive cation permeability or the number of sodium pumps present in these cells or alterations in the characteristics of the sodium pumps present.

The present work was therefore directed towards characterising the Na^+/K^+ activation curves of the sodium pump in cattle red cells with values for internal K^+ ranging from 20–100 mmoles/l cells. For convenience, the ouabain-sensitive ATPase activity of fragmented red cell ghosts was chosen to investigate the internal affinity of the pump for K^+ [5]; unidirectional ouabain-sensitive K^+ uptake measurements were also made. It is concluded that cattle red cell K^+ levels are determined by the variable internal affinity of the red cell sodium pump for K^+.

METHODS

Blood

Blood was taken into heparin by jugular venapuncture from cattle of the Jersey herd at Babraham. Cells were washed three times by centrifugation (5 min, $3000 \times g$) in 10 vol. of 107 mM $MgCl_2$, 10 mM Tris–HCl pH 7.6 at 20 °C.

Preparation of ghosts

Ghosts were prepared by hypotonic lysis in 14 vol. of 10 mM Tris–HCl, 1 mM EDTA pH 7.5 at 4 °C, followed by centrifugation (30 min, $25\,000 \times g$) and three successive washes, one in the Tris–HCl–EDTA medium, and two with 15 vol. of 10 mM Tris–HCl pH 7.5 at 4 °C. After freezing and thawing the fragmented membranes were washed once in 10 mM Tris–HCl, 0.1 mM EDTA pH 7.5 at 4 °C, and then finally in 10 mM Tris–HCl pH 7.5 at 4 °C.

ATPase assay

Membranes were incubated for 1–2 h at 37 °C at a concentration equivalent to 25 % original packed cells in 1.4 ml of a medium containing 1.25 mM $MgCl_2$, 1.25 mM Na_2-ATP, 10 mM Tris–HCl pH 7.7 at 37 °C, 145 mM Na^+ and varying K^+ or choline concentrations from 0–100 mM. The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 5 %, and inorganic phosphate determine by a modification of the Weil-Malherbe and Green method [12]. (Na^+–K^+) activated ATPase was measured as the ouabain-sensitive component by the selective addition of ouabain to a final concentration of 10^{-4} M. All conditions were assayed in quadruplicate.

K^+ uptake

Washed red cells were incubated for 1 h at a haematocrit of 5 % in a medium containing 154 mM NaCl, 2 mM $MgCl_2$, 5 mM glucose, 10 mM Tris–HCl pH 7.7 at 37 °C, and varying concentration of KCl (containing ^{42}KCl) and choline chloride. The cells were washed four times by centrifugation (3 min, $3000 \times g$) in ice-cold 107 mM $MgCl_2$, 10 mM Tris–HCl pH 7.5 at 4 °C, lysed and processed for γ counting and oxyhaemoglobin determination.

K^+ levels

Intracellular K^+ and Na^+ was determined by flame photometry following lysis and dilution of cells washed in the isotonic $MgCl_2$ medium.

Anti-L

The anti-L antibody was raised in an HK sheep to homozygous LK (LL)

sheep red cells as previously described [13]. Serum, partially absorbed with various HK sheep cells, was precipitated by dialysis into 15 % Na_2SO_4, washed in the Na_2SO_4 solution by centrifugation (3 min, 3000 × *g*) dialysed against 10 mM Tris–HCl pH 8.0, filtered and freeze dried. Fragmented ghosts were sensitized with the antibody by incubation, at a haematocrit equivalent to 10 % original packed cells, with 10 mg/ml of the freeze dried anti-serum in 10 mM Tris–HCl pH 7.7 at 32 °C for 30 min. The ghosts were washed twice in 30 vol. of 10 mM Tris–HCl pH 7.5 at 4 °C, before use.

RESULTS

The total and ouabain-sensitive ATPase activity of fragmented ghosts from a cow with an original red cell K^+ level of 50 mmoles/l cells is illustrated in Fig. 1. The biphasic shape of the ouabain-sensitive ATPase curve (Fig. 1a) is similar to that from LK sheep and goats, and is consistent with an initial stimulation by K^+ acting at the external sites with an apparent affinity of < 2 mM ($Na^+ = 150$ mM), followed by a K^+ inhibition at higher K^+ levels consistent with a high affinity for K^+ as an inhibitor at the internal Na^+ loading sites. Although from curve 1b, where the ouabain-insensitive ATPase component remains invariant with changes in KCl concentration, it seems unlikely that indirect effects of high K^+ concentration inhibiting the action of ouabain were involved. A control experiment was designed to check that ouabain was exerting its maximal effect even at 100 mM K^+ (Table 1). No significant differences were observed between 1 and 0.01 mM ouabain at either 5

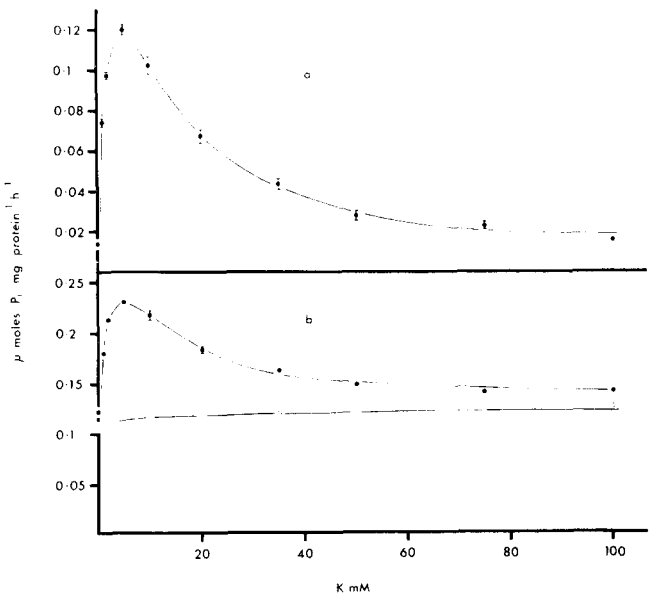

Fig. 1. ATPase activity in cow erythrocyte ghost membranes as a function of K^+ concentration. (a) Ouabain-sensitive ATPase activity. (b) ●–●, total and, ○–○, + ouabain (10^{-4} M) ATPase activity. Error bars represent ±S.E. (4 duplicates), curves drawn by eye. Incubation medium contains 145 mM NaCl, 1.25 mM $MgCl_2$, 1.25 mM Na_4-ATP, 10 mM Tris–HCl pH 7.7 at 37 °C, 100 mM (KCl + choline chloride). Original red cell K^+, 50 mmoles/l cells.

TABLE I

THE EFFECT OF VARYING OUABAIN CONCENTRATIONS ON THE (Na^+–K^+)-ACTIVATED ATPase ACTIVITY OF CATTLE RED CELLS

Additions	ATPase activity (μmoles P_i/mg protein per h)		
	K^+ = 0 mM	K^+ = 5 mM	K^+ = 100 mM
None	0.107±0.002	0.221±0.002	0.172±0.001
10^{-3} M ouabain	0.100±0.002	0.100±0.002	0.097±0.001
10^{-5} M ouabain	0.101±0.002	0.099±0.001	0.101±0.001

Fragmented ghosts from cow M2, K^+ = 96 mmoles/l cells.

or 100 mM K^+. In all the experiments, adding ouabain gave a significantly lower activity than removing K^+, consistent with an ouabain-sensitive Na^+-dependent ATPase being present.

The affinity for K^+ of the external K^+ site was determined independently by K^+ uptake experiments on red cells from individual cattle with different internal K^+ levels. Fig. 2 presents the ouabain-sensitive K^+ uptake for red cells from two cows with red cell K^+ of 77 and 24 mmoles/l cells. Although there is a large difference in the maximum rates of influx, the K^+ concentration for half-maximal uptake is very similar for both, at 0.78 and 0.96 mM, respectively. Similar experiments were carried out exploring the ouabain-sensitive ATPase activation over the K^+ concentration range 0–10 mM confirming the similarity of the K^+ activation phase irrespective of the original cell K^+. It therefore seems that, in high Na^+ media, the apparent external

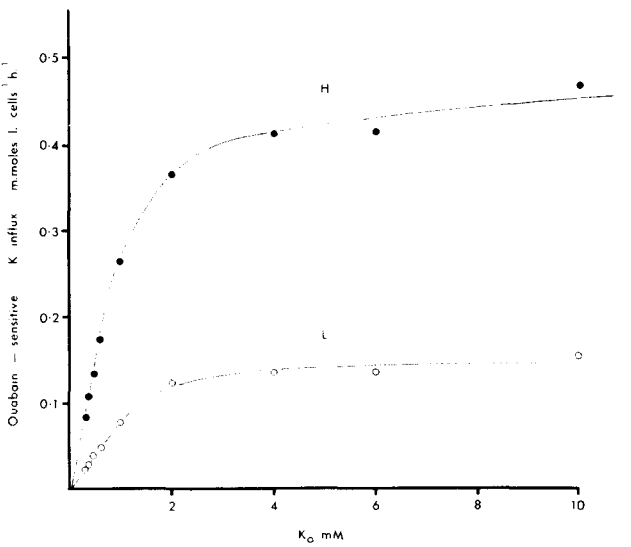

Fig. 2. Ouabain-sensitive K^+ uptake by red cells from two cows with high or low intracellular K^+. Upper curve (H) for animal K^+ = 77 mmoles/l cells; lower curve (L) for animal K^+ = 27 mmoles/l cells. Cells incubated in 154 mM NaCl, 10 mM KCl (containing ^{42}KCl)+choline Cl, 2 mM $MgCl_2$, 5 mM glucose, 10 mM Tris–HCl pH 7.7 at 37 °C. S.E. (4 duplicates) smaller than the symbols, curves drawn by eye.

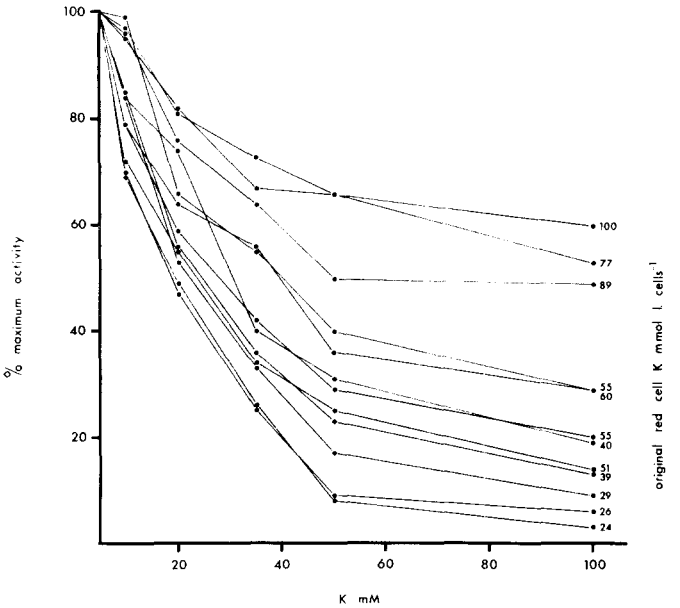

Fig. 3. Ouabain-sensitive ATPase activity in fragmented ghosts from cows with differing red cell K^+ levels. Data normalized for 5 mM K^+ = 100 %; Na^+ = 150 mM; ouabain, when present, 10^{-4} M. Original red cell K^+ indicated in mmoles/l cells to the right of each individual curve.

K^+ affinity of the sodium pump in cattle red cells is about constant, even for cells of differing internal concentrations. Similar results in high Na^+ media were found for sheep [4], and goats [14].

To compare directly the shape of the internal K^+ inhibition curves for fragmented ghosts from individuals with differing red cell K^+ levels, ouabain-sensitive ATPase, determined as a function of increasing K^+ (> 5 mM) at Na^+ = 150 mM, was expressed as a fraction of the activity at K^+ = 5 mM, the condition for approximate maximum activity. The results of these determinations are given in Fig. 3. It is clear that the degree of K^+ inhibition is related to the original red cell K^+ level, ranging from 90 % inhibition at high K^+ levels in ghosts from red cells with $K^+ < 30$ mmoles/l cells, compared with less than 50 % inhibition in cells where $K^+ > 70$ mmoles/l cells. One striking feature is the absence of any clear identification of two (corresponding to "HK" and "LK") or three (corresponding to MM (HK)), ML (heterozygous LK) and LL (homozygous LK)) types of enzyme. Rather, from the sample of 26 animals so far investigated it seems that a continuous spectrum of K^+ sensitivities exists.

Control experiments, in which red cells from the same animal are investigated at intervals over a six month period, revealed that the internal K^+ level and K^+-dependent ATPase characteristics remained a constant feature.

The specific sheep blood group antibody, anti-L, has been shown to stimulate active K^+ transport in LK sheep and goat red cells, at least partly by changing the internal affinity for potassium [5, 6, 8]. Although the reaction of anti-L with cattle red cells is more variable, there is a consistent stimulation of active K^+ influx in red

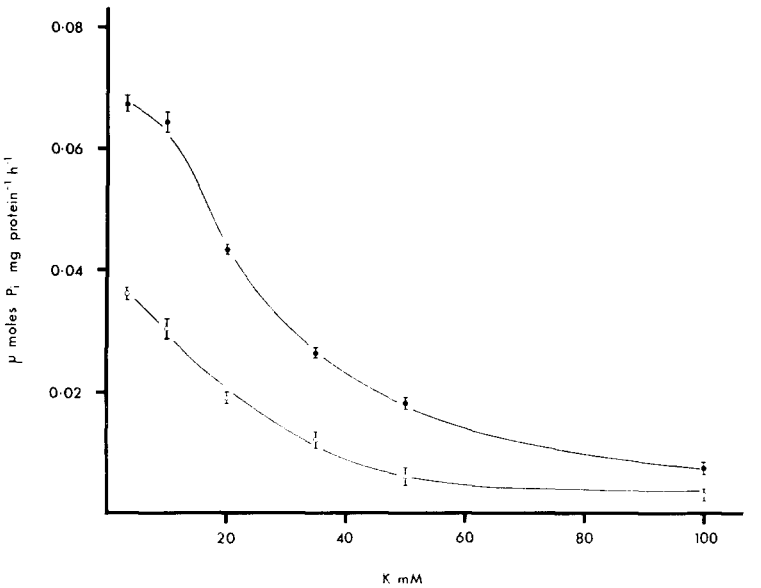

Fig. 4. The effect of anti-L on the K^+-activation curve of the ouabain-sensitive ATPase in cattle erythrocytes. Original red cell K^+, 29 mmoles/l cells. ○–○, control; ●–●, anti-L treated cells; Na^+ = 150 mM; ouabain, when present, 10^{-4} M. Values given as ±S.E. (4 duplicates).

cells from most individual cattle by certain batches of anti-L. When this interaction was investigated by measuring ATPase activity (Fig. 4), it is clear that anti-L does interact with cattle red cells, increasing the maximal ATPase activity, and probably slightly decreasing the internal affinity for K^+. However the stimulation is less dramatic than that observed in sheep and goats, and this suggests that anti-L may only be interacting with a fraction of the sites available.

DISCUSSION

The present results confirm several previous studies which demonstrate an ouabain-sensitive ATPase activity in cattle red cells [10, 15, 16]. However, the K^+ inhibition characteristics reported in this paper make it important to define K^+ concentrations carefully when measuring levels of ATPase activity in ruminant red cell ghosts, particularly when comparing "HK" and "LK" type cells [10], or assessing changes in ATPase levels in calf red cells [16] during maturation.

From measurements of either red cell K^+ levels, the K^+ dependence of (Na^+–K^+)-activated ATPase, or anti-L binding, it is easy to define sheep erythrocytes as HK or LK type. The present work was therefore started with the premise that "HK" or "LK" type enzyme might exist in cattle red cells, with variations in the amount of enzyme present. In practice, a complete spectrum of K^+ inhibition curves was obtained, indicating large individual variations in the properties of the ouabain-sensitive ATPase. These characteristics could be a product of differing proportions of HK and LK type enzymes being present either within the same cell, or less likely, in different cells in the same individual. If this were the case, and even cells with high (60–80

mmoles/l cells) levels of K^+ have a small amount of LK-type enzyme, it would explain the failure of all attempts to produce an anti-L by injecting "LK" type cattle cells into an "HK" cow [11]. It could also account for the partial and variable results often obtained in the reaction of different batches of anti-L with cattle red cells.

It is known that red cells from the newborn and reticulocytes from the anaemic animal in LK type sheep and cattle have a high internal K^+ and increased Na^+ pump activity [13, 16–20]. Recently, several authors [6, 16, 21, 22] have speculated on the possibility that mature LK red cells may have a population of "degenerate" Na^+ pumps, and maturation of the original HK type cells to LK type cells, which is associated with the operational activity of the Lp antigen leads to a conversion of active K^+ or Na^+ transport into passive transport. Evidence for this has been inferred from experiments on ouabain binding [21], the effects of anti-L on ouabain-insensitive K^+ fluxes [6, 21], and $Na^+ : Na^+$ exchange and passive fluxes [6, 16, 22]. The present data, indicating a spectrum of K^+ sensitivities in sodium pumps in individual cattle are not incompatible with this suggestion, where the K^+-inhibited pump could still carry out "degenerate" functions. As in other ruminant red cells, in cattle the Lp antigen seems to be associated with LK characteristics of the sodium pump. If all immature cattle cells are originally HK type, variation in the amount of antigen affecting the Na^+ pumps during maturation could lead to a mixed population of Na^+ pumps in the mature situation. Clearly, detailed studies on the production and operational activity of the Lp antigen during erythropoiesis are necessary to pursue this study further.

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