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A CHANGE IN THE INTERNAL AFFINITY OF LK GOAT RED-CELL SODIUM PUMPS INDUCED BY HIGH pH

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Summary

The K inhibition of ouabain-sensitive ATPase activity of LK goat red cell membranes is greatly reduced at high pH. This effect is reversible, and specific, since the apparent affinities for ATP, ouabain or external K do not alter. Anti-L-treated membranes show a similar alkali-induced affinity change, but have a lower pH optimum.

Introduction

The sodium pump in low potassium (LK) red cells of ruminants differs in certain kinetic properties from the normal sodium pump found in most other cells. In particular, whilst experiments with human cells have shown that the internal ion-binding aspect of the sodium pump can be described kinetically in terms of 3 equivalent sites with about a 45-fold higher affinity for Na than K at each of the sites (i.e. K_m^{Na} = 0.2 mM; K_i^K = 9 mM) [1], in contrast LK goat red cells show a 6-fold higher internal affinity for K than Na (i.e. K_m^{Na} = 20 mM; K_i^K = 3 mM), [2,3]. This kinetic modification is related to the presence on the cells of a specific antigen, designated L, since reaction of the anti-L antibody with the cells alters the internal affinities in favour of sodium [2—6].

In this paper we demonstrate a significant change in the apparent internal affinity for K in LK cells at a high pH, mimicking the effect of anti-L. Ouabain-sensitive (Na + K)-activated ATPase activity was chosen as the most convenient experimental situation for demonstrating these effects, since although it removes the asymmetry of the ion distribution in intact cells (which could be preserved by using the *p*-chloromercuribenzene sulphonate or nystatin methods) it allows more convenient manipulation of the assay conditions, and yields essentially similar results (e.g., compare ref. 3 with ref. 6).

Methods

Blood

Blood was taken from 2 LK Saanen goats (cell K = 29 and 34 mmol/l cell water^{-1}) by jugular venepuncture into heparinized containers. The cells were washed 3 times by centrifugation (3000 × *g* for 5 min) and stored in a medium containing 150 mM NaCl, 10 mM glucose, 15 mM Tris, pH 7.6 at 20°C.

ATPase measurements

Fragmented ghosts were prepared by lysing washed red cells in 15 volumes of 10 mM Tris, 1 mM EDTA, pH 7.4 at 4°C, followed by 2 washes by centrifugation (25 000 × *g* for 30 min). The pellet was washed twice more in 10 mM Tris, pH 7.4, at 4°C, and stored quick-frozen at −20°C, at a volume equivalent to the original packed cell volume.

ATPase activity was assayed in 1.2 ml of a solution containing (usually) NaCl 150 mM, KCl 5 mM, $MgCl_2$ 2 mM, ATP 2 mM, with 20 mM buffer. The ghost concentration was 10% packed cell equivalent, with incubations for 1—2 h at 37°C. Samples were run in triplicate, with parallel incubations in the presence of ouabain (0.15 mM). The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 5%, and the inorganic phosphate liberated was assayed by the method of Fiske and Subbarow [7], modified to use amidol as reducer. Where K concentrations were varied no adjustment was made for ionic strength, since preliminary experiments using isosmotic choline chloride replacement revealed no effect. Where ATP varied, $MgCl_2$ levels were also altered to keep the Mg : ATP ratio at 1. The buffers normally used were 2-(*N*-morpholino)ethanesulphonic acid (MES) (pH 6—7), *N*-Tris-(hydroxymethyl)methyl-2-aminoethane-sulphonic acid (TES) (pH 7—8) or Tris (pH 8—10). The pH of the incubation was checked just before terminating the incubation. The ouabain-sensitive fraction of ATPase activity was normally 50—70% of the total when assayed at Na 150 mM, K 5 mM, pH 7.5, with normal values for the ouabain-sensitive component in the range 0.4—0.8 μmol/ml packed cells per h.

K influx

Ouabain-sensitive K influx was measured by incubating cells at 3.5% haematocrit in a medium containing NaCl 130 mM, KCl (including ^{42}K) 7.5 mM, glucose 10 mM, buffer 25 mM, ± 0.15 mM ouabain for 1 h at 37°C. The reaction was stopped in an ice-bath, the cells washed 4 times in 15 volumes of ice-cold 107 mM $MgCl_2$, 10 mM Tris, pH 7.5 at 4°C in a microcentrifuge (10 000 × *g* for 15 s), lysed in 5% trichloroacetic acid and the supernatant counted for Cerenkov radiation. The haematocrit was estimated by measuring A_{541} of lysed cell suspensions.

Anti-L treatment

Anti-L was prepared as previously described [8], and purified by absorptions and Na_2SO_4 precipitation [9]. Cells were sensitized by incubation at a haematocrit of 5% with antibody at a concentration of 10 mg/ml total IgG at 32°C for 30 min. This was followed by a single wash. When assayed as ouabain-sensitive ATPase activity, the anti-L-treated membranes did not always show an increased activity at K = 5 mM, but were stimulated 1.6- to 2.4-fold at K = 20 mM.

Results

Fig. 1 shows the pH optimum for the ouabain-sensitive ATPase activity of LK goat red cells, before and after sensitization with the anti-L antibody. The data are for a single incubation condition (K 5, Na 150), chosen for optimum activity at the usual pH, 7.5. Results have been expressed as a percentage of the highest activity achieved. It will be seen that LK cells (the solid line) have a maximum in the region of pH 8—8.5, and show significant activity at higher pH values. In contrast, anti-L-treated membranes (the broken line) show an optimum at pH 7.5, and are 90% inhibited by pH 9. Three different buffers, MES, TES and Tris were used to cover the range of this experiment. To control for possible specific effects of a particular buffer both MES and TES were compared at pH 7, and TES and Tris at pH 8, for the solid line in Fig. 1. Since essentially identical results were achieved, no major specific buffer effect seems likely.

(Na + K)-activated ATPase is a complicated multi-ligand enzyme, and it is therefore important to assess whether this pH effect is on the maximum velocity of the reaction, or rather involves a change in the affinity for a particular ligand (Na or K inside or out; ATP; Mg; ouabain).

Fig. 2 shows the effect of increasing K concentrations on the ouabain-sensitive ATPase activity measured at three different pH values. Again the data are expressed as a fraction of the activity obtained at K = 5 mM. It is clear that increasing pH has a marked effect on the apparent K affinity with pH 9 showing a greatly reduced K inhibition. At K > 5 mM, the principal site at which K is

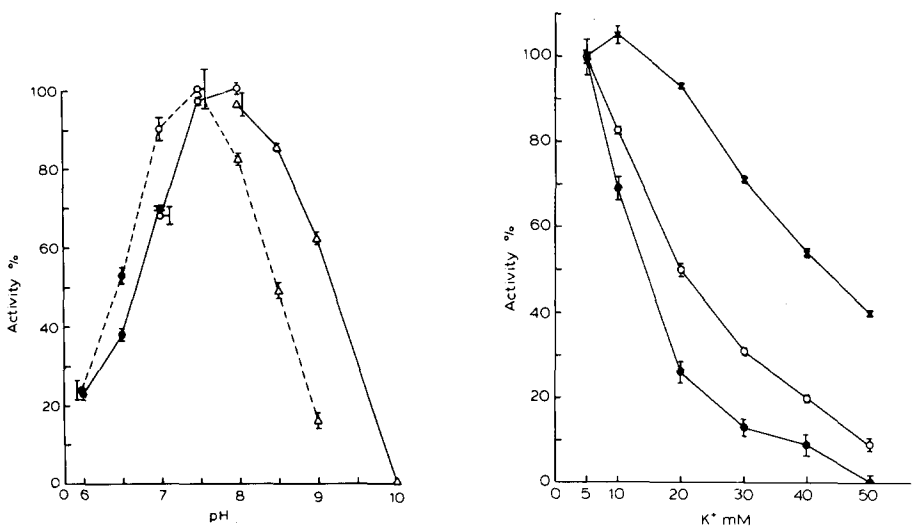

Fig. 1. The effect of pH on the (Na + K)-activated, ouabain-sensitive ATPase activity of LK goat red cell membranes. ———, normal cells; - - - - - -, anti-L-treated cells. •, MES; ○, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES); △, Tris. Maximal measured activity (= 100%) control 0.700, anti-L-treated 0.577, ouabain-insensitive 0.83 μmol P_i/ml cells per h. Error bars are S.E.M.

Fig. 2. The effect of pH on the K inhibition of ouabain-sensitive ATPase activity of LK goat red cell membranes. •, MES; ○, HEPES; X, Tris, pH 6.0, 7.5, and 9.0 respectively. Measured maximal activities (= 100%) 0.143, 0.735 and 0.508 μmol P_i/ml cells per h at pH 6, 7.5 and 9. Error bars are S.E.M.

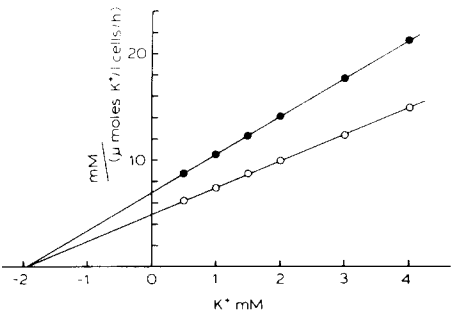

Fig. 3. Concentration dependence of ouabain-sensitive K influx in LK goat red cells measured at pH 7.5 and 9. ○, pH 7.5; ●, pH 9.0. Data presented as S/V versus S plot. Line drawn by eye.

acting is internal, since the external K site has an apparent K_m^K of $\approx$ 2.0 mM under these conditions. However, to confirm this point, Fig. 3 presents data for the ouabain-sensitive K influx into LK cells at pH 7.5 and 9, plotted in the form S/V against S. Since the two lines give essentially the same intercept on the X-axis, it can be seen that there is no signicant change in the external K affinity over this pH range.

Fig. 4 a and b directly compares the effect of increasing concentrations of K on the ouabain-sensitive ATPase activity in normal and anti-L-treated LK membranes at pH 7.5 and 9. It will be seen that at both pH values anti-L still causes a shift in the affinity away from K, and again LK membranes at pH 9 also show a decreased affinity for K. These data have been normalized to 100% = K 5 mM to compare the shapes of the curves. The actual activities measured are given in the legend to Fig. 4 and show that although the K inhibition has been removed,

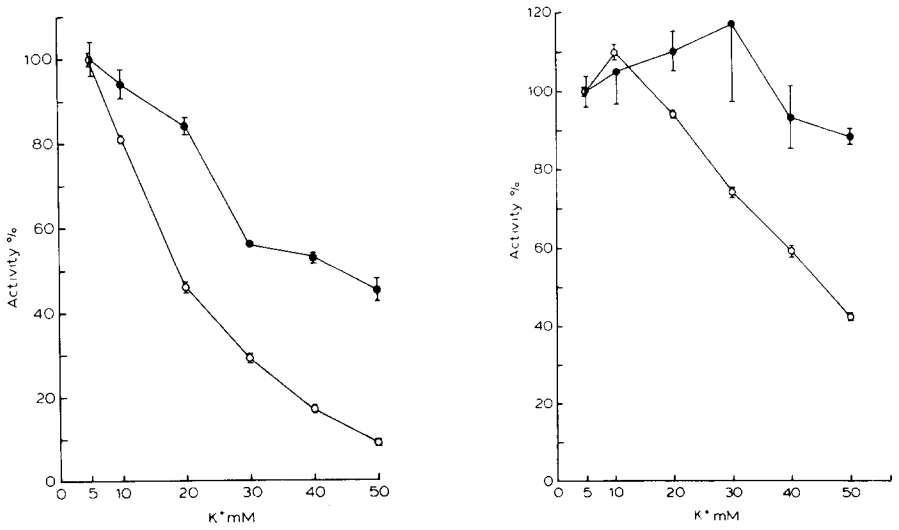

Fig. 4. The effect of anti-L on the K inhibition of the ouabain-sensitive ATPase activity of LK goat red cell membranes at pH 7.5 (a) and 9 (b). ○, control; ●, anti-L. Measured maximal activities control 0.847 (a) 0.437 (b) anti-L 0.724 (a) 0.153 (b) μmol P_i/ml cells per h. Error bars are S.E.M.

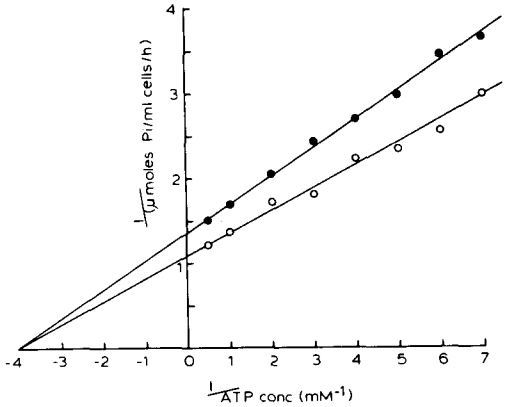

Fig. 5. ATP concentration dependence of ouabain-sensitive ATPase activity of LK goat red-cell membranes measured at pH 7.5 and 9.0. ○, pH 7.5; ●, pH 9.0. Data presented in the form $1/V$ versus $1/S$. Lines drawn by eye.

the absolute activity of the anti-L-treated enzyme at pH 9 is only 20% of that at pH 7.5 (cf. Fig. 1).

Further experiments at varying ATP concentrations showed that no significant change in the apparent ATP affinity for the pH change 7.5 to 9 occurred in this system (Fig. 5). A further important control experiment was to establish that the ouabain-sensitivity of the preparation was not altering with increasing pH, since we rely on this as the index of ATPase inhibition. Table I presents data for 3 different ouabain concentrations at the two relevant pH values: and confirms that the inhibition seems maximal, and does not alter over this pH range.

One question of interest was whether this pH effect was reversible. An experiment in which cells were preincubated at pH 7.5 or 9 for 30 min and then washed and assayed at the complementary pH was therefore carried out (Table II). It can be seen (upper half of Table II) that the results were identical irrespective of the preincubation conditions. A further finding of this experiment (lower half of Table II) was that anti-L could successfully bind at pH 9, i.e. the action of high pH in mimicking anti-L with regard to the internal K affinity was not removing the anti-L binding site.

TABLE I

THE EFFECT OF pH ON THE OUABAIN-SENSITIVITY OF LK GOAT RED-CELL MEMBRANE ATPase

Ouabain concentration (mM)	ATPase activity (µmol P_i/ml cells per h)	
	pH 7.5	pH 9.0
0	1.448 ± 0.016	1.322 ± 0.005
0.014	0.703 ± 0.003	0.725 ± 0.001
0.14	0.700 ± 0.003	0.722 ± 0.007
1.4	0.706 ± 0.000	0.722 ± 0.002

TABLE II

THE EFFECT OF PRE-INCUBATION AT pH 7.5 OR 9.0 ON THE ($Na^+ + K^+$)-ATPase ACTIVITY OF LK GOAT RED-CELL MEMBRANES SUBSEQUENTLY ASSAYED AT pH 7.5 OR 9.0

	Pre-incubation pH	Assay pH	Ouabain-sensitive ATPase activity (μmol/ml per h)	
			K5	K20
Control	7.5	7.5	0.74 ± 0.02	0.35 ± 0.02
	9.0		0.67 ± 0.01	0.34 ± 0.01
	7.5	9.0	0.49 ± 0.01	0.46 ± 0.01
	9.0		0.46 ± 0.01	0.41 ± 0.01
Anti-L	7.5	7.5	1.13 ± 0.02	0.89 ± 0.02
	9.0		0.92 ± 0.01	0.70 ± 0.02
	7.5	9.0	0.55 ± 0.03	0.67 ± 0.01
	9.0		0.42 ± 0.02	0.46 ± 0.01

Discussion

The sodium pump in LK cells is uniquely different from the normal pump in showing a high internal affinity for K. This effect is due to the presence of the L antigen on the cells, associated with the pump. Although the antibody-binding site is only accessible from the outside surface, anti-L has virtually no effect on the external Na and K affinities, but exerts its effect internally by changing the ratio of $K_m^{Na} : K_i^K$ from 6 to 2 [2,3]. In the present work we have demonstrated an equivalent effect by increasing the pH from 7.5 to 9. This appears to be specific for the internal Na-loading site since there are no marked changes in the affinity for K_0, ATP or ouabain.

Very little is known of the chemistry of the L antigen which has so far eluded attempts to isolate or purify it [e.g., see ref. 10]. Preliminary radiation inactivation studies indicate that L is separate from the pump itself, and has an apparent molecular size in the range $3—7 \cdot 10^5$ daltons (Ellory, J.C., unpublished observations). Degradation studies with proteolytic enzymes on whole cells have revealed that the external anti-L binding site can be removed by trypsinization [11], but we have recently shown (Ellory, J.C. and Beauge, L., unpublished observations) that internally, trypsin can have the same effect as high pH or anti-L, e.g., can decrease the internal K affinity. The conclusion at the present must be that the L antigen is a large molecule spanning the membrane and interacting with the internal binding site of the sodium pump. The active inhibitory group can be reversibly titrated by high pH to reduce the effectiveness of the L antigen.

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