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A K^+-DEPENDENT PHOSPHATASE IN THE MEMBRANES OF LOW-K^+-TYPE ERYTHROCYTES

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SUMMARY

1. A small K^+-dependent fraction of *p*-nitrophenylphosphatase activity has been demonstrated in LK-type ruminant red cell ghosts.

2. The K^+-dependent enzyme has a K_m of about 3.3 mM for *p*-nitrophenylphosphate, and shows substrate inhibition.

3. This phosphatase is stimulated by the anti-L antibody, without any measurable change in the apparent affinity for K^+.

4. Addition of 20 mM $Na^+ + 10\,\mu M$ ATP causes an increase in phosphatase activity at low concentrations, followed by inhibition as the K^+ concentration is increased. Anti-L treated cells show an increased ($Na^+ + ATP$)-dependent stimulation but without change in the reactivity towards K^+.

5. The K^+-sensitive phosphatase is inhibited 50% by 1 mM ouabain in the absence, and 90% in the presence of ($ATP + Na^+$).

A K^+-dependent, ouabain-sensitive acylphosphatase (EC 3.6.1.7) was found in close association with the ($Na^+ + K^+$)-dependent ATPase (EC 3.1.6.3) from almost any source tested [e.g. 1, 2]. An interesting exception seems to be the ($Na^+ + K^+$)-ATPase obtained from low-potassium-type (LK) erythrocyte membranes of sheep and cattle, in which the K^+-sensitive phosphatase was reported to be absent [3–5]. This could reflect a basic important difference in the molecular arrangement of LK-type Na^+ pumps, since the K^+-dependent phosphatase is supposed to reflect the ability of the "normal" ($Na^+ + K^+$)-ATPase to hydrolyse other phosphate esters apart from the phosphorylated intermediate, its natural substrate [6].

We have now been able to demonstrate the existence of a K^+-dependent, ouabain-sensitive *p*-nitrophenylphosphatase activity in the membrane of LK-type erythrocytes from sheep, cattle and goats. We report here some preliminary experiments on the kinetic properties of the K^+-dependent LK goat phosphatase which show characteristic similarities and differences with the HK-type human enzyme.

Goat blood was drawn into heparin, washed three times in 20 vol. of a solution containing 107 mM $MgCl_2$ and 10 mM Tris–HCl (pH 7.6 at 20 °C), and lysed at 4 °C in 14 vol. of a solution containing 10 mM Tris–HCl and 1 mM $Tris_4EDTA$. The membranes were washed twice by centrifugation ($25000 \times g$, 10 min) in this medium, frozen and thawed quickly and washed 3 times more in 10 mM Tris–HCl.

TABLE I

THE *p*-NITROPHENYLPHOSPHATASE ACTIVITY OF LOW-K^+ TYPE RUMINANT RED CELL MEMBRANES

Data for HK goat and human red cells, and the $(Na^+ + K^+)$-dependent ATPase are included for comparative purposes.

Red cell membranes from	*p*-Nitrophenylphosphatase (mmoles/l cells per h)			$(Na^+ + K^+)$-activated ATPase (mmoles/l cells per h)*	Ratio of K^+-*p*-nitrophenylphosphatase / $(Na^+ + K^+)$-ATPase
	$-K^+$	$+K^+$	K^+-induced difference		
LK cow	0.0994 ± 0.0014	0.1193 ± 0.0013	0.0199 ± 0.0019	—	—
LK sheep	0.1271 ± 0.0005	0.1406 ± 0.0017	0.0135 ± 0.0018	—	—
LK goat	0.1483 ± 0.0014	0.1693 ± 0.0007	0.0210 ± 0.0016	0.23 ± 0.02	0.1
HK goat	0.152 ± 0.002	0.432 ± 0.004	0.280 ± 0.005	0.68 ± 0.02	0.4
Human**	0.26 ± 0.05	0.59 ± 0.06	0.33 ± 0.08	0.67 ± 0.11	0.5

* Assayed as previously described [8].

** Data from Viglíocco et al. [3].

In order to measure the *p*-nitrophenylphosphatase activity, a volume of ghosts equivalent to 0.12 ml of original packed cells was incubated, usually for 4 h at 37 °C, in 1.1 ml of a medium containing 6 mM $MgCl_2$, 5 mM *p*-nitrophenylphosphate (di-Tris salt); 5 mM Tris–HCl buffer (pH 7.9) at 37 °C, and 20 mM KCl. When the concentration of K^+ was varied, equimolar concentrations of Tris–HCl or choline chloride were used to replace the KCl. Other changes in the medium are mentioned in the legends to figures and tables. The reaction was stopped by the addition of 0.2 ml of 33% trichloroacetic acid. After centrifugation, 1 ml of supernatant was added to 2 ml 0.2 M NaOH and the concentration of *p*-nitrophenol determined spectrophotometrically at 410 nm. All volumetric procedures were performed with Oxford samplers or Krogh-type calibrated syringes and at least five duplicates were used for each condition. The standard error of each determination was kept well within 2% of the total value. Preliminary experiments showed that the hydrolysis of *p*-nitrophenylphosphate was linear with time during the 4 h incubation. The maximum hydrolysis of *p*-nitrophenylphosphate represented less than 2% of the initial amount of substrate present inside each tube.

Anti-L serum was raised as previously described [7]. The serum was dialysed against 4 vol. of 19% Na_2SO_4, overnight at 25 °C and the precipitate washed 3 times in 20 vol. of 15% Na_2SO_4. It was finally redissolved in 10 mM Tris–HCl buffer, extensively dialysed against 10 mM Tris at 4 °C, centrifuged and the supernatant freeze dried. Ghosts were treated with the antibody by incubation for 30 min at 32 °C in 5 vol. of antibody, 15 mg/ml in 10 mM Tris–HCl buffer, followed by two washes in 50 vol. of 10 mM Tris–HCl buffer.

Table I gives the values of *p*-nitrophenylphosphatase activity measured in the ghosts obtained from sheep, cow and goat LK red cells. It can be seen that the K^+-sensitive component represents a very small proportion of the total phosphatase activity. Under comparable conditions this proportion seems to be genuinely lower in LK than in HK or human red cell membranes. The same applies to the ratio of K^+-dependent phosphatase to $(Na^+ + K^+)$-ATPase.

Fig. 1 shows the substrate dependence of the *p*-nitrophenylphosphatase in the presence and absence of saturating K^+ concentrations. In the absence of K^+, the Mg^{2+}-phosphatase shows a complex behaviour as a function of *p*-nitrophenylphosphate concentration. The presence of K^+ changes this situation very little and the reactivity of the phosphatase remains much the same, since the maximum stimulation induced by K^+ is less than 10% of the total phosphatase activity. The behaviour of the K^+-sensitive component, on the other hand, follows a simple saturation curve with characteristic substrate inhibition, a feature in common with the human red cell enzyme [9]. It therefore seems more fruitful to analyse the LK phosphatase in terms of a K^+-sensitive and a K^+-insensitive component, the former showing maximum reactivity towards specific cofactors of the $(Na^+ + K^+)$-ATPase, rather than consider the total phosphatase as a single enzyme in two different reactive forms, depending of the presence or absence of K^+ [9].

When the concentration of substrate is kept constant, the K^+-sensitive phosphatase saturates with increasing K^+ concentrations (Fig. 2; control). A specific sheep blood group antibody, anti-L, which stimulates K^+ transport and the $(Na^+ + K^+)$-ATPase of LK cells [10], also stimulates the K^+-sensitive *p*-nitrophenylphosphatase activity, without changing its affinity for K^+ (Fig. 2; anti-L). It is not yet

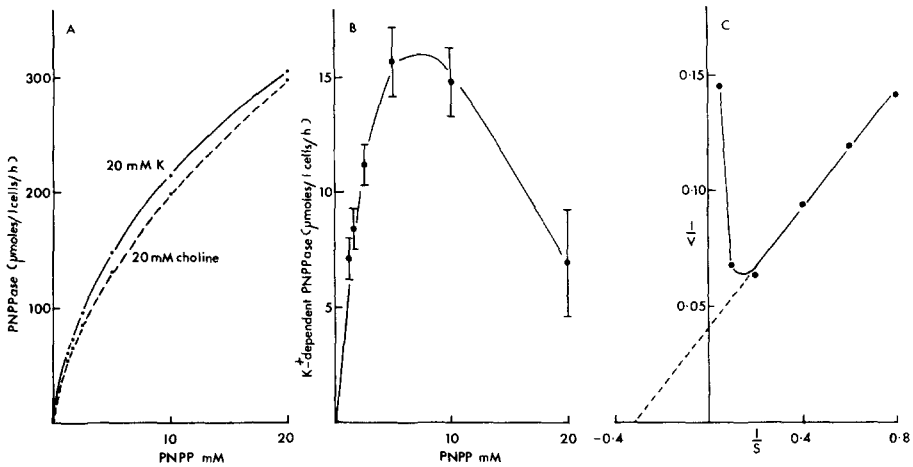

Fig. 1. A. The *p*-nitrophenylphosphatase (PNPPase) activity of LK goat red cell membranes as a function of the concentration of *p*-nitrophenylphosphate (PNPP), in the presence and absence of K^+. *p*-Nitrophenylphosphate was replaced by $Tris_2SO_4$ so that the total concentration of *p*-nitrophenylphosphate and $Tris_2SO_4$ was 20 mM. The variation in the concentration of SO_4^{2-}-induced changes in the concentration of Mg^{2+}. However, preliminary experiments showed that both the K^+-sensitive and the K^+-insensitive components saturated at Mg^{2+} concentrations lower than the lowest estimated value of Mg^{2+}: 1.7 mM (in the presence of 20 mM of SO_4^{2-}). $Tris_2SO_4$ was chosen since it was necessary to replace *p*-nitrophenylphosphate without altering the ionic strength of the medium. The LK phosphatase is moderately inhibited by increasing the ionic strength in a similar way to the human red cell enzyme [20, 21]. This is also the reason why the LK *p*-nitrophenylphosphatase was always tested at the lowest ionic strength compatible with the desired conditions of measurement. The standard error corresponding to each point is within the size of the point on the ordinate scale. B. This curve represents the difference between the phosphatase activity in the presence and absence of K^+ (Fig. 2A). C. Lineweaver–Burk plot of the K^+-dependent *p*-nitrophenylphosphatase of Fig. 2B showing characteristic substrate inhibition.

clear whether the antibody acts by unmasking new LK or HK type pumps or by changing the reactivity of LK-type pumps towards Na^+ and K^+ bringing them nearer to the kinetic behaviour of HK-type pumps thereby increasing the turnover rate of the individual pumps [11–16]. The site at the internal surface of LK pumps which normally binds and translocates Na^+, shows a higher affinity for K^+, which here acts as an inhibitor, than for Na^+, the normal translocating substrate [16]. Anti-L seems to increase the affinity of Na^+ relative to that of K^+ but it is not yet clear whether this action involves a change in affinity for both ions or only for one of them. The fact that anti-L stimulates the phosphatase without changing the apparent K_m for K^+ is difficult to reconcile with an action mediated through a decrease in the affinity for K^+ at its inhibitory internal site. This point was investigated further by looking at the action of the antibody in the simultaneous presence of Na^+ and ATP. Under these conditions, the human red cell phosphatase shows a large increase in the affinity for external K^+ together with a decrease in the V for K^+ activation of the phosphatase [17–19]. If a similar effect is induced on the affinity for K^+ in LK cells, then one might expect a stimulatory action of Na^++ATP at low K^+ concentrations followed by inhibition when the K^+ is raised. This is indeed the case as can be seen in Fig. 2 (control; ATP+Na^+). There is, however, a characteristic difference from the human

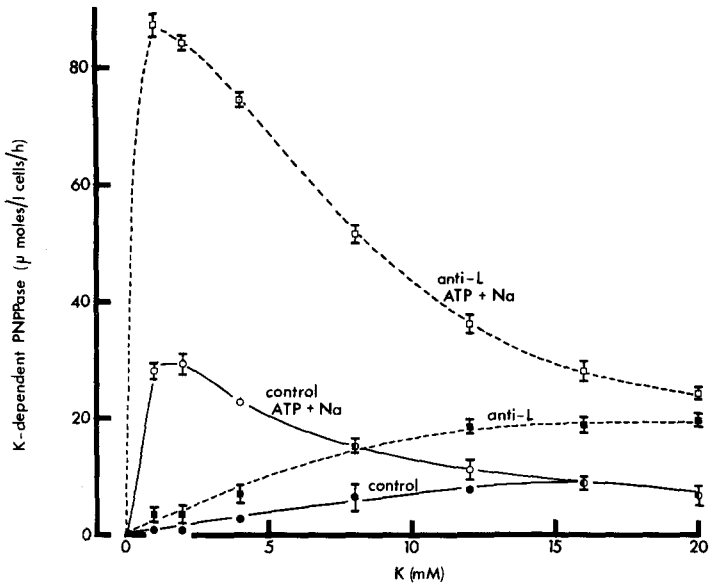

Fig. 2. K^+-sensitive LK goat red cell *p*-nitrophenylphosphatase (PNPPase) as a function of the concentration of K^+. ($ATP+Na^+$) curves were obtained in the presence of 20 mM NaCl, 10 μM of $Tris_4ATP$, 5 mM creatine phosphate, di-Tris salt, and creatinephosphokinase, 0.10 mg/ml in addition to the standard medium referred to in the text. The presence of the ATP-regenerating system was necessary in order to avoid changes in the concentration of ATP due to enzymic hydrolysis. The presence of the buffer system probably had no effect in itself as shown in control experiments. It is important to remember, when comparing curves in the presence of ($ATP+Na^+$) with curves in their absence, that the ionic strength of the (Na^++ATP) medium is higher than the standard medium and, therefore, represents a relatively inhibited value. Anti-L curves report the phosphatase activity obtained in ghosts pretreated with anti-L as detailed in the text.

red cell enzyme; the *V* of the K^+-stimulated enzyme is much larger in the presence of Na^+ +ATP than in its absence. However the more significant effect is that anti-L, while stimulating the overall phosphatase activity in the presence of Na^++ATP, did not

TABLE II

THE EFFECT OF 10^{-3} M OUABAIN ON THE PHOSPHATASE ACTIVITY OF LK GOAT RED CELL MEMBRANES

Additions		*p*-Nitrophenylphosphatase activity (μmoles/l cells per h)		Ouabain inhibition (%)
		−ouabain	+ouabain	
None	K^+, 0	175.5±0.9	178.0±0.6	
	K^+, 10 mM	189.2±0.5	184.4±0.6	
	Difference	13.7±1.0	6.4±0.9	50
Na^++ATP*	K^+, 0	159.4±0.3	160.4±0.3	
	K^+, 2 mM	191.2±0.3	164.4±0.9	
	Difference	31.8±0.4	4.0±1.0	88

* Conditions as described in Fig. 2.

decrease appreciably the inhibitory effect of K^+ (Fig. 2; anti-L; ATP+Na^+). If anti-L reduces the affinity for K^+ at the inhibitory site when its action is explored on the ($Na^+ + K^+$)-ATPase and on the K^+ influx [15, 16] but not on the phosphatase activity then a second inhibitory site for K^+ must be involved in its effect on this enzyme. At the moment, however, one cannot exclude other possibilities like small variations in the action of different batches of antibody. Ideally, ATPase and phosphatase activity should be measured simultaneously as a function of K^+. Preliminary results indicate that this might be possible since ATP is only a partial inhibitor of the LK phosphatase.

Table II shows the effect of a single ouabain concentration on the K^+-dependent *p*-nitrophenylphosphatase, in the presence and absence of Na^++ATP. In common with the human red cell phosphatase [9] it can be seen that the ouabain sensitivity is largely enhanced by the presence of Na^++ATP. Ouabain had no effect in the absence of K^+ and, at this concentration, inhibited the K^+-sensitive component only partially.

The measurement of the LK phosphatase opens new ways in the investigation of the molecular properties of LK-type Na^+ pumps and will probably throw light on the basic differences between the pumps of LK and HK erythrocytes.

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