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Acylphosphatase induced modifications in the functional properties of erythrocyte membrane sodium pump

Paolo Nassi, Elena Marchetti, Chiara Nediani, Gianfranco Liguri and Giampietro Ramponi

Dipartimento di Scienze Biochimiche, Università di Firenze, Florence (Italy)

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Human red cell acylphosphatase actively hydrolyzes the Na^+/K^+-ATPase phosphoenzyme from erythrocyte membrane. This effect occurred with amounts of acylphosphatase (up to 10 units/mg membrane protein) within the physiological range, and the low value of the apparent K_m (0.147 ± 0.050 μM) indicates that the enzyme has a high affinity for this substrate. When added at the above concentration to inside out vesicles from human erythrocytes, acylphosphatase significantly enhanced the rate of strophantidine-sensitive ATP hydrolysis. The same amounts of acylphosphatase stimulated, although to a lower extent, the rate of ATP-dependent $^{22}Na^+$ influx (normal efflux). Thus, the calculated stoichiometry for Na^+/ATP was 2.68 in the absence of acylphosphatase and 1.06 in the presence of 10 units/mg vesicle protein of the enzyme. Conversely, acylphosphatase addition strongly decreased the rate of ATP-dependent $^{86}Rb^+(K^+)$ efflux (normal influx) which, with 10 units/mg vesicle protein, was almost suppressed. As a consequence, the Na^+/Rb^+ ratio, calculated as 1.52 in the absence of acylphosphatase rose to 72.5 in the presence of 10 units/mg vesicle protein of this enzyme. These results suggest that, because of its hydrolytic activity on the phosphoenzyme intermediate, acylphosphatase 'uncouples' erythrocyte membrane Na^+,K^+ pump. Possible mechanisms for this effect are discussed.

Introduction

Most mammalian cells have in their plasma membrane Na^+,K^+ pumps which transport Na^+ ions out of and K^+ ions into the cell. This transport process requires energy and, in fact, the Na^+,K^+ pump may be considered as an energy transducer converting chemical energy from the hydrolysis of ATP to an electrochemical gradient which in turn represents a free energy source for many important cellular processes.

The Na^+,K^+ pump, identified as a Na^+/K^+-dependent membrane-bound ATP hydrolyzing enzyme, hence the name of Na^+/K^+-ATPase, is an electrogenic system since, under normal conditions, for each ATP hydrolyzed, three Na^+ ions are transported out of and two K^+ ions into the cell [1]. Despite numerous studies concerning the structural and functional properties of Na^+/K^+-ATPase, the molecular mechanism for the action of this membrane pump is still debated. There is no doubt, however, that the transient formation of an acylphosphorylated phosphoenzyme (EP) intermediate, through a Na^+-dependent phosphorylation and a K^+-dependent dephosphorylation of a specific aspartyl residue in the α-subunit of Na^+/K^+-ATPase, is a crucial event for the production of the conformational transitions underlying the changes in affinity for the two cations and their transport coupled to ATP hydrolysis [2].

Acylphosphatase (EC 3.6.1.7) is a widespread cytosolic enzyme that catalyzes the hydrolysis of the carboxylphosphate bond of acylphosphates, such as 3-phosphoglyceroyl phosphate [3], carbamoyl phosphate [4] and succinoyl phosphate [5]. For several years we have been studying structural and functional properties of acylphosphatase purified to homogenity from different sources. The results thus obtained lead us conclude that in mammalian tissues acylphosphatase is present in two isoenzymatic forms: one is prevalent in skeletal and cardiac muscle, the other in red blood cells (RBCs); the RBC isoenzyme, apart from slight

Correspondence to: P. Nassi, Dipartimento di Scienze Biochimiche Università di Firenze, Viale Morgagni 50, 50134 Florence, Italy.

Abbreviations: RBC, red blood cell; Na^+/K^+-ATPase, sodium-potassium ion-dependent adenosine triphosphatase; Ca^{2+}-ATPase, calcium ion-dependent adenosine triphosphatase; P_i, inorganic phosphate; IOV, inside out vesicle of red cell membrane; SDS, sodium dodecyl sulphate; E, the Na^+/K^+-ATPase enzyme; EP, the phosphorylated form of E; E_1 and E_2, conformational forms of E; E_1P and E_2P, conformational forms of EP.

differences in the affinity towards substrates and sensibility to inhibitors, exhibits higher catalytic potency when compared to the muscular isoform [6]. Recently, we found that RBC acylphosphatase, in addition to the above soluble, low molecular weight substrates, can actively hydrolyze the acylphosphorylated intermediates of Ca^{2+}-ATPase [7] from human erythrocyte membrane.

In this paper we describe the results of experiments aimed to evaluate whether a similar effect of acylphosphatase on the phosphoenzyme intermediate of Na^+/K^+-ATPase resulted in altered functional properties of this system in terms of both ATP hydrolysis and cation transport.

Materials and Methods

Reagents were obtained from Merck, except Tris-ATP and Dextran T-70 (M_r 72600) which were obtained from Sigma. $^{22}NaCl$ and $^{86}RbCl$ were from New England Nuclear.

[γ-^{32}P]ATP (6000 Ci/mmol) was purchased from the Radiochemical Centre, Amersham.

Acylphosphatase was purified from human erythrocytes according to Degl'Innocenti et al. [8]; the enzyme, isolated as a pure product, had a specific activity of 7500 units/mg protein using benzoyl phosphate as substrate [9]. Benzoyl phosphate was synthesized according to Camici et al. [10].

Membrane protein content was assayed with the biuret method of Beisenherz et al. [11] using bovine serum albumin as a standard.

Preparation of inside out vesicles from RBC membranes

Inside-out vesicles (IOVs) from red cell membrane, prepared by the method of Blostein [12], were separated by layering 2 ml of needled vesicles on the top of 3 ml of Dextran T-70 solution (1.115 g/25 ml 0.5 mM Tris-P_i, pH 8) and centrifuging for 1 h at 48000 rpm in a Beckman SW 50.1 Ti rotor. The vesicles banding at the top of the dextran solution were washed once in 10 mM Tris-glycylglycine (pH 7.4) and two more times in 20 mM Tris-glycylglycine (pH 7.4). The vesicles were suspended in the latter buffer at a concentration of 3–4 mg protein/ml and stored at 4°C for a period up to 1 week. The efficiency of our IOV preparation (as judged by acetylcholinesterase accessibility) was 82%.

Preparation of Na^+/K^+-ATPase ^{32}P-phosphoenzyme and its incubation with acylphosphatase

IOVs were used to prepare Na^+/K^+-ATPase ^{32}P-phosphoenzyme using a modification of the method of Kuwayama H. and Kanazawa T. [13]. 1 ml of reaction mixture contained 100 mM NaCl, 1 mM EGTA, 5 mM $MgCl_2$, 1 μCi [γ-^{32}P]ATP, 0.15 M Tris-HCl (pH 7.2) and 1.5 mg vesicle protein. The phosphorylation reaction, carried out at 0°C, was started by the addition of non-radioactive Tris-ATP to give a final concentration of 15 μM ATP. The reaction was stopped, after 5 s, by adding 5 vol. of ice-cold stop solution containing 6% trichloroacetic acid, 1 mM ATP and 5 mM NaH_2PO_4. The phosphorylated vesicles were centrifuged at 30000 $\times g$ for 10 min. Pellets were resuspended, washed once in the above stop solution and three additional times in ice-cold 0.15 M Tris-HCl (pH 7.2). The amount of ^{32}P bound to vesicles was determined by counting the radioactivity of an aliquot of the pellet, dissolved in 3% SDS, in a liquid scintillation mixture. The phosphoenzyme level was calculated by subtracting nonspecific ^{32}P bound to vesicles phosphorylated by the same procedure as that described above, except that 100 mM KCl was used in place of NaCl.

Labeled vesicles (2 mg protein/ml) were incubated in 0.150 M Tris-HCl (pH 7.2) at 37°C with differing amounts of acylphosphatase, as indicated in Table I, for 1 min; the reaction was terminated by adding 2 vol. of final 6% trichloroacetic acid, and the suspensions were centrifuged at 12000 $\times g$ for 5 min. Aliquots of the supernatants were used for measuring free ^{32}P radioactivity. Controls for spontaneous hydrolysis of the phosphoenzyme were incubated under the same conditions, except that acylphosphatase was omitted. In other experiments differing amounts of vesicles were

TABLE I

Effect of different acylphosphatase concentration on the release of phosphate from RBC membrane Na^+/K^+-ATPase phosporylated intermediate

Labeled membranes (2 mg protein/ml assay medium) were incubated in 0.150 M Tris-HCl (pH 7.2) at 37°C for 1 min with varying amounts of acylphosphatase. The phosphoenzyme level was calculated as 66.0 ± 1.6 pmol phosphate bound/mg membrane protein. (A) Phosphate released from vesicles labeled in the presence of Na^+. (B) Phosphate released from vesicles labeled without Na^+. Results are means ± S.E. of six experiments. All the changes in phosphate release induced by active acylphosphatase in column A were statistically significant ($P < 0.01$ by one-way analysis of variance). Heat-inactivated acylphosphatase (2 h at 100°C) was added at a concentration of 1.33 μg (corresponding to 10 units of active enzyme) per mg membrane protein.

Acylphosphatase addition	Phosphate release (pmol/mg protein per min)	
	A	B
None (control)	36.46 ± 1.73	2.31 ± 0.26
0.5 units/mg	45.24 ± 1.40	2.25 ± 0.23
1 units/mg	50.37 ± 1.10	
2 units/mg	55.55 ± 1.85	2.42 ± 0.36
5 units/mg	59.93 ± 2.18	
10 units/mg	63.96 ± 1.98	2.44 ± 0.15
Heat-inactivated	37.18 ± 1.00	

incubated in the same conditions above described with a fixed amount of acylphosphatase.

Measurements of Na^+ transport, Rb^+ transport and ATP hydrolysis

For all these experiments, vesicles were first equilibrated overnight at 4°C and then preincubated for 30 min at 37°C with 20 mM Tris-glycylglycine (pH 7.4), 50 mM choline chloride, 1 mM $MgSO_4$, 0.5 mM RbCl, except for the Rb^+ transport assay where vesicles were equilibrated with 0.5 mM ^{86}RbCl (10 mCi/mmol).

Na^+ transport was measured as ^{22}Na influx into IOVs by the filtration procedure described elsewhere [14]. Assays of Na^+ transport were carried out at 30°C in a medium containing 45 mM choline chloride, 5 mM ^{22}NaCl (1.6 MCi/mmol), 1 mM $MgSO_4$, 0.7 mg IOV protein/ml, 20 mM Tris-glycylglycine (pH 7.4) in the presence or in the absence of 50 μM Tris-ATP.

Rb^+ transport was measured as the ATP-dependent ^{86}Rb efflux from preloaded IOVs. This was determined evaluating ^{86}Rb retained in the vesicles by the above mentioned filtration method. Assays for Rb^+ transport were performed at 30°C in media identical to those used for Na^+ influx except that 5 mM non-radioactive NaCl was used.

Na^+/K^+-ATPase activity was measured as the rate of strophantidine-sensitive ATP hydrolysis [15]. Vesicles were assayed at 30°C in 20 mM Tris-glycylglycine (pH 7.4), 45 mM choline chloride, 5 mM NaCl, 1 mM $MgSO_4$, 0.05 ouabain, 0.02 μCi [γ-^{32}P] ATP and 50 μM Tris-ATP. When present, strophantidine was added to the IOV suspension in the last 5 min of preincubation at 37°C at the final concentration of 0.1 mM. The hydrolysis reaction was terminated by adding 0.9 ml of an ice-cold solution containing 5% trichloroacetic acid, 5 mM Na_2ATP and 2.5 mM NaH_2PO_4 to 0.1 ml of reaction volume.

Following centrifugation, at 12000 × g for 5 min, 0.9 ml of supernatants were removed and 0.45 ml of 5% trichloroacetic acid containing charcoal (15 gr/100 ml) was added.

The suspensions were kept on ice for about 1 h and then centrifuged at 12000 × g for 10 min. An aliquot of the clear supernatants was used for measuring free ^{32}P radioactivity.

In all the experiments on cation transport and ATP hydrolysis, acylphosphatase, when present, was added at a concentration of 2, 5, 7, 10 units/mg IOV protein.

Expression of the results

Data presented the Results are the means of several determinations and statistical analysis was performed using the Student's t-test or, where indicated, by one-way analysis of variance. Curves were drawn on the basis of the mean values with a computer data analysis program, Enzfitter by Elsevier Biosoft, version 1.03.

Results

To prepare the Na^+/K^+-ATPase phosphoenzyme erythrocyte membrane IOVs were phosphorylated with [γ-^{32}P]ATP as described under Materials and Methods. Under these conditions the phosphate associated to the Na^+,K^+ pump was, on average, 66 pmol/mg IOV protein.

To study the effect of acylphosphatase on the Na^+/K^+-ATPase phosphoenzyme (EP), we measured the phosphate released by vesicles incubated with varying amounts of acylphosphatase, from 0.5 to 10 units/mg membrane protein. Such concentrations were chosen because they are within or near the physiological range which, on the account of acylphosphatase activity in human RBCs and of protein content of human erythrocyte membrane, was estimated to be 3–7 units/mg membrane protein [16]. As shown in Table I, when acylphosphatase was added to the vesicles phosphorylated in the presence of Na^+, the release of phosphate bound was costantly higher than spontaneous hydrolysis and rose significantly with the increase in acylphosphatase concentration. On the other hand, heat-inactivated acylphosphatase did not produce significant enhancement of phosphate release with respect to the spontaneous dephosphorylation. The spontaneous release of phosphate from the vesicles phosphorylated in the absence of Na^+ was very low and it was not modified by acylphosphatase addition even with the active form. Taken together these data indicate that acylphosphatase can actively hydrolyze the Na^+/K^+-ATPase phosphoenzyme which in the present conditions, represents the only substrate for our enzyme among the phosphorylated components of the erythrocyte membrane.

In other series of experiments, intended to evaluate the affinity of our enzyme towards EP, variable amounts of vesicles were incubated with a fixed amount (2 units) of acylphosphatase. Under these conditions (Fig. 1) we found that the inital rate of acylphosphatase-induced dephosphorylation, measured as a function of EP concentration, rose along a hyperbolic curve with an apparent K_m of 0.147 ± 0.050 μM.

To determine whether the above described action on the phosphoenzyme intermediate resulted in modified functional properties of Na^+/K^+-ATPase, we examined the effects of acylphosphatase on the rate of Na^+,Rb^+-dependent ATP hydrolysis and on ATP-dependent cation (Na^+, Rb^+) transport. In all these studies we used Rb^+ as substitute for K^+ and erythrocyte membrane IOVs as a source of Na^+/K^+-ATPase as it exists in situ. Such vesicles, that we obtained in a good yield in terms of the sideness, allowed us to perform quantitative studies on ion fluxes and ATP hydrolysis under identical conditions. In accordance with Harvey and Blostein [15], the ATPase activity due

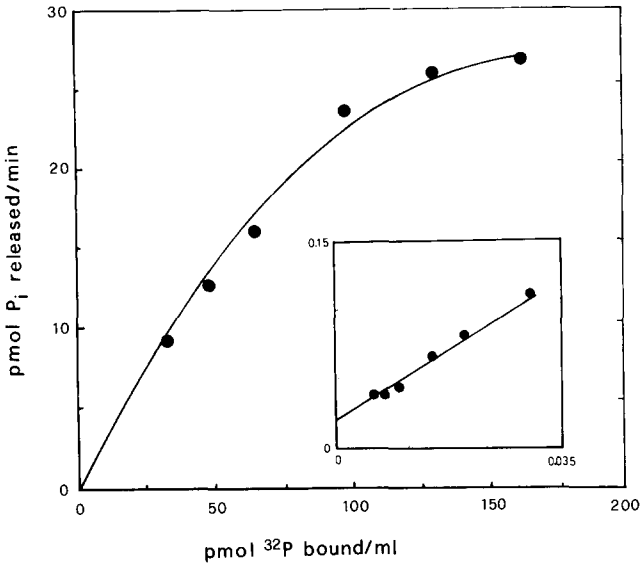

Fig. 1. Initial rate of Na^+/K^+-ATPase intermediate dephosphorylation by acylphosphatase. 2 units of acylphosphatase were incubated in 0.150 M Tris-HCl (pH 7.2) at 37°C with different amounts of labeled membranes. Values are corrected for spontaneous hydrolysis. EP concentration in the medium was expressed as pmol of ^{32}P bound/ml. Each point represents the mean value of five determinations. The inset shows the double-reciprocal plot of the data shown in the figure.

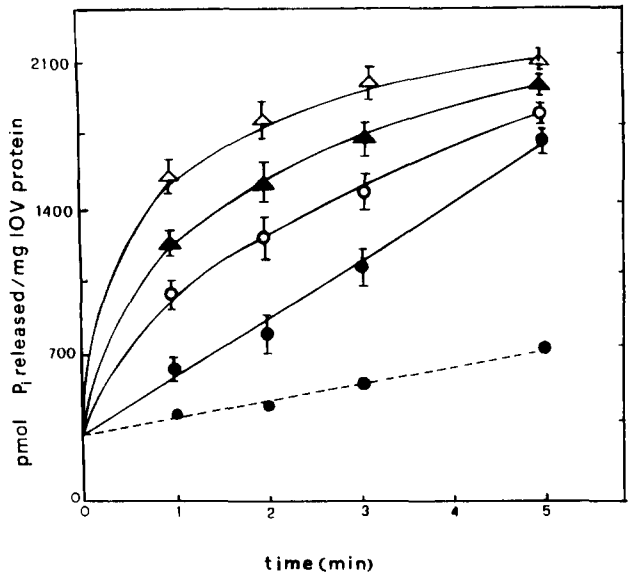

Fig. 2. Time course of Na^+/K^+-ATPase activity in control and acylphosphatase-incubated IOVs. IOVs were equilibrated and assayed as described under Materials and Methods. Assays were carried out at 30°C in a medium containing 20 mM Tris-glycylglycine (pH 7.4), 45 mM choline chloride, 5 mM NaCl, 1 mM $MgSO_4$, 0.7 mg IOV protein/ml, 0.02 μCi [γ-^{32}P]ATP and 50 μM Tris-ATP in the absence (●) and in the presence of varying amounts of acylphosphatase (○ 2 U/mg, ▲ 5 U/mg, △ 10 U/mg IOV protein). The dotted line indicates the ATPase activity in the presence of 0.1 mM strophantidine. Ouabain (0.05 mM) was present in all media to inhibit Na^+/K^+-ATPase activity of broken or permeable membranes. Each point represents the mean ± S.E. of five determinations.

to the Na^+,K^+ pump was calculated as the difference in the rate of ATP hydrolysis without and with strophantidine which, being membrane permeant, was added to inhibit total glycoside sensitive activity; furthermore ouabain was present in all assays to inhibit the activity of right side, permeable or broken vesicles. Under these conditions the strophantidine-sensitive activity was considered as the expression of the Na^+/K^+-ATPase activity of unleaky IOVs.

The effect of acylphosphatase on Na^+/K^+-ATPase activity is illustrated in Fig. 2 and Table II. From Fig. 2, where the time courses of phosphate release from ATP are reported, it is evident that acylphosphatase strongly accelerated the strophantidine-sensitive ATP hydrolysis and this effect was more marked with increasing amounts of our enzyme. Table II shows that the changes induced by acylphosphatase in the initial rate of strophantidine-sensitive ATPase were statistically significant and maximal stimulation, obtained with 10 U/mg IOV protein, resulted in an activity which was more than 5-fold the basal value (1091 ± 58 against 187 ± 20 pmol/min per mg IOV protein, mean values ± S.E.). No appreciable effect was observed using heat-inactivated acylphophatase.

Acylphosphatase effects on cation transport were studied by measuring, both in the absence and in the presence of different amounts of the enzyme, the ATP-dependent Na^+ influx into IOVs, equivalent to the normal efflux from the cell, and the ATP -dependent Rb^+ (substitute for K^+) efflux from loaded IOVs,

TABLE II

Effect of acylphosphatase on strophantidine-sensitive Na^+/K^+-ATPase activity of RBC membrane inside-out vesicles

IOVs were equilibrated as described under Materials and Methods. Strophantidine-sensitive Na^+/K^+-ATPase activity was assayed in a medium containing 20 mM Tris-glycylglycine (pH 7.4), 45 mM choline chloride, 5 mM NaCl, 1 mM $MgSO_4$, 0.7 mg IOV protein/ml, 0.05 mM ouabain, 0.02 μCi [γ-^{32}P]ATP, 50 μM ATP. Each value is the difference in the mean ± S.E. of four determinations performed on vesicles incubated with and without strophantidine. Changes observed with differing amounts of active acylphosphatase were statistically significant ($P < 0.01$). Heat-inactivated acylphosphatase (2 h at 100°C) was added at a concentration of 1.33 μg (corresponding to 10 U of the active enzyme) per mg IOV protein.

Acylphosphatase addition (U/mg IOV protein)	Strophantidine-sensitive Na^+/K^+-ATPase activity (pmol/min per mg IOV protein)	Increase *n*-fold
None	187 ± 20	1
2	521 ± 61	2.79
5	779 ± 53	4.16
7	902 ± 43	4.83
10	1091 ± 58	5.83
Heat-inactivated	195 ± 15	1.04

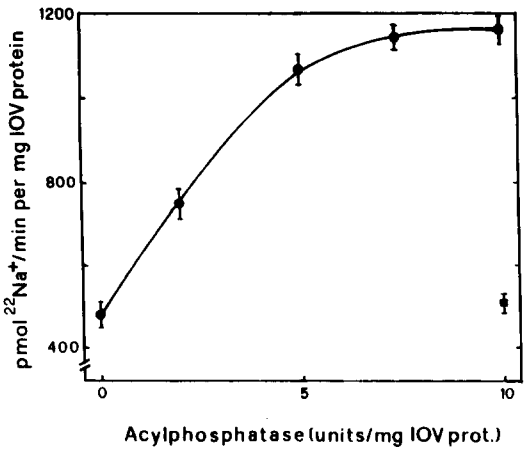

Fig. 3. Effect of acylphosphatase on $^{22}Na^+$ transport into IOVs. IOVs were equilibrated as described under Materials and Methods. ATP-dependent $^{22}Na^+$ uptake was measured at 30°C in a medium containing 5 mM $^{22}NaCl$, 45 mM choline chloride, 1 mM $MgSO_4$, 0.7 mg IOV protein/ml with or without 50 μM Tris-ATP. At 30-s intervals over a 2-min period 100-μl samples were added to 10 ml of a stop solution containing 45 mM choline chloride, 5 mM NaCl, 1 mM $MgSO_4$, 20 mM Tris-glycylglycine (pH 7.4). $^{22}Na^+$ uptake was measured by the filtration technique described elsewhere [14]. ■ indicates the value obtained with inactivated acylphosphatase (1.33 μg corresponding to 10 units of the active enzyme). Values are corrected for ATP-independent $^{22}Na^+$ transport. Each point represents the mean ± S.E. of four determinations. Changes observed with varying amounts of active acylphosphatase resulted in statistical significance ($P < 0.01$).

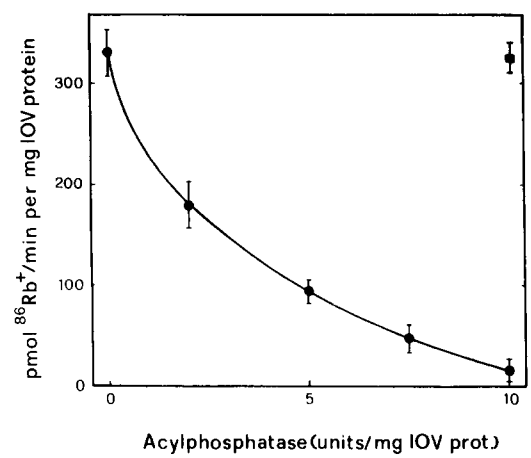

Fig. 4. Effect of acylphosphatase on $^{86}Rb^+$ loss from RBC membrane inside out vesicles. IOVs were equilibrated as described under Materials and Methods. ATP-dependent $^{86}Rb^+$ transport was measured at 30°C in a medium containing 5 mM NaCl, 45 mM choline chloride, 1 mM $MgSO_4$, 0.7 mg IOV protein/ml and 20 mM Tris-glycylglycine (pH 7.4) with or without 50 μM Tris-ATP. At 30-s intervals over a 2-min period 100-μl samples were added to 10 ml of the stop solution (see Fig. 3) and $^{86}Rb^+$ retention was measured by the filtration technique described elsewhere [14]. ■ indicates the value obtained with 1.33 μg (corresponding to 10 U of active enzyme) of inactivated acylphospatase (2 h at 100°C). Values are corrected for ATP independent $^{86}Rb^+$ transport. Each point represents the mean ± S.E. of four determinations. All changes observed with active acylphosphatase resulted statistically significant ($P < 0.01$).

equivalent to the normal influx into the cell. Measurements were taken at 30-s intervals over a 2-min period and values observed without ATP were substracted in order to exclude the contribution by other, non-active, transport systems (such as ion channels). In the absence of acylphosphatase Na^+ uptake proceeded linearly throughout the above time at a rate of 502 ± 15 (S.E) pmol/min per mg IOV protein. When acylphosphatase was added to the system, we observed that the rate of ATP-dependent Na^+ uptake was significantly stimulated and more markedly so with higher enzyme amounts (Fig. 3). With 10 units/mg IOV protein Na^+ uptake was more than 2-fold the basal value.

Conversely, acylphosphatase addition resulted in a dramatic decrease in the rate of Rb^+ loss from loaded IOVs (Fig. 4). Under our experimental conditions the ATP-dependent Rb^+ efflux, without acylphosphatase, was 330 ± 25 (S.E.) pmol/min per mg IOV protein. A remarkable reduction was observed even with the lowest acylphosphatase concentration; with 10 units per mg IOV protein the active Rb^+ transport was almost suppressed, being only 16 ± 3 (S.E.) pmol/min per mg

TABLE III

Effect of acylphosphatase on Na^+/ATP and Na^+/Rb^+ stoichiometry

Strophantidine-sensitive Na^+,K^+-ATPase activity, ATP-dependent $^{22}Na^+$ influx (normal efflux) and $^{86}Rb^+$ efflux (normal influx), expressed as pmol/per mg IOV protein, were assayed at 30°C as described in Table II, Figs. 3 and 4. Reactions were carried out for 1 min with 50 μM ATP. Each value shown is the mean ± S.E. of four determinations and all changes induced by acylphosphatase were statistically significant ($P < 0.01$).

Acylphosphatase addition (U/mg IOV protein)	Strophantidine-sensitive Na^+/K^+-ATPase activity (pmol/mg per min)	ATP-dependent ^{22}Na influx (pmol/mg per min)	ATP-dependent ^{86}Rb efflux (pmol/mg per min)	Na^+/ATP ratio	Na^+/Rb^+ ratio
None	187 ± 20	502 ± 14	330 ± 25	2.68	1.52
2	521 ± 61	746 ± 29	181 ± 24	1.43	4.12
5	779 ± 53	1071 ± 25	95 ± 8	1.37	11.27
7	902 ± 43	1144 ± 21	43 ± 7	1.27	26.60
10	1091 ± 58	1160 ± 19	16 ± 3	1.06	72.50

vesicle protein. No significant modifications in active Na^+ and Rb^+ transport were observed using heat-inactivated acylphosphatase.

As a consequence of all the above effects, acylphosphatase addition appears to produce a modification in the stoichiometry of the ATP driven cation transport by the Na^+,K^+ pump. The Na^+/ATP and Na^+/Rb^+ ratios calculated with and without acylphosphatase are reported in Table III.

Discussion

The present study provides evidence that acylphosphatase can actively and specifically hydrolyze the phosphorylated intermediate of RBC membrane Na^+,K^+ pump. This result is added to our previous findings that indicated a similar acylphosphatase effect on the phosphoenzyme of erythrocyte membrane Ca^{2+} pump [7].

Although it was the acid-denaturated EP which was subjected to hydrolysis by our enzyme (but admittedly it is difficult or impossible to perform experiments with native EP), we think it is important to emphasize the fact that the acylphosphatase effect was observed in RBC membranes which were not subjected to disruption or other drastic treatment and when using this enzyme at concentrations within the physiological range.

It may be also noteworthy that the measurement of the initial rate of acylphosphatase-induced hydrolysis as a function of Na^+,K^+ pump phosphoenzyme concentration gave an apparent K_m value in the order of 10^{-7} M, which suggest a high affinity in our enzyme for this special substrate. This might be consistent enough with the low number of copies of Na^+,K^+ pump in RBC membrane, all the more so because the K_m values that we observed for erythrocyte acylphosphatase towards other potentially competing substrates, including a variety of low molecular weight cytosolic components, were constantly higher than 10^{-4} M [17].

In addition to the above action on the phosphoenzyme intermediate, we found that acylphosphatase markedly affects the functional properties of Na^+,K^+ pump, notably the rate of ATP hydrolysis and of cation (Na^+,Rb^+) transport.

In order to compare the effects on ATP hydrolysis and pumping activity, all these experiments, performed using erythrocyte membrane IOVs, were conducted under the same experimental conditions regarding the temperature and the concentrations of extravesicular Na^+, intravesicular Rb^+ and ATP. Although the ATP concentration (50 μM) may be a somewhat low concentration compared with the generally reported K_m values for ATP of Na^+/K^+-ATPase, the described conditions were chosen after several trial experiments, because they appeared to be the most suitable to ensure a good efficiency of the pump, at the same time preventing a too rapid loss of Rb^+ from vesicles [14]. In fact, our system, without added acylphosphatase,

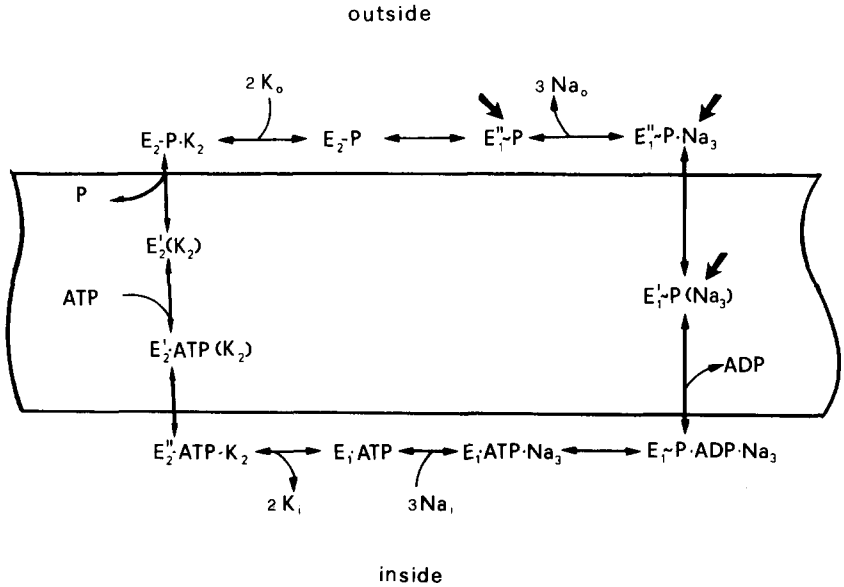

Fig. 5. A scheme for Na^+–K^+ exchange by Na^+/K^+-ATPase. The scheme is based on that reported by Skou [1]. E_1 and E_2 refer to different conformations of the enzyme, E_1 representing the form with high Na^+-low K^+ affinity, E_2 the form with high K^+-low Na^+ affinity. E, E′ and E″ represent transient conformational states and EP the phosphorylated intermediate. Parentheses indicate that the cations are occluded, i.e., in the membrane phase inside gates closed toward the cytoplasmic and the extracellular medium. Bold arrows indicate the supposed sites of acylphosphatase action.

exhibited a Na^+/ATP ratio near to the well-documented value of 3:1 and also the stoichiometry of ATP-dependent Na^+/Rb^+ exchange was consistent with the value of 3:2 observed under normal conditions [18–22]. When the rate of ATP hydrolysis and of cation transport were measured in the presence of acylphosphatase we observed different effects on these parameters, since both ATPase activity and Na^+ uptake were stimulated, but the latter to a much lower extent. Conversely, the ATP-dependent Rb^+ efflux from IOVs was strongly inhibited. All the effects of acylphosphatase, which does not exhibit per se ATPase activity, were more marked with increasing amounts of this enzyme. Furthermore, as for the hydrolysis of the phosphoenzyme, heat-inactivated acylphosphatase did not produce appreciable modifications in ATPase and in pumping activity, nor did the active enzyme have significant effects (data not shown) on the ATP-independent Na^+ influx and Rb^+ efflux from vesicles. Thus, it may be excluded either that acylphosphatase acts rather like a protein than an enzyme or that the effects of acylphosphatase on Na^+ and Rb^+ transport are simply due to alterations in the passive permeability of RBC membrane for these cations.

On the contrary, the present results indicate that acylphosphatase, in its catalytically active form, exerts a sort of 'uncoupling' effect on RBC membrane Na^+,K^+ pump, as it is evident from the altered Na^+/ATP and, especially, Na^+/Rb^+ ratios, that we observed when the enzyme was added.

For a possible interpretation of the acylphosphatase effect, one may consider the most widely used models to describe the functioning of Na^+,K^+ pump. On the assumption that the reactions with Na^+ and K^+ are consecutive, the sequence of chemical reactions, conformational transitions and cation transport may be depicted by the scheme shown in Fig. 5. This scheme is derived from that reported by Skou [1], who combined and modified other schemes previously proposed by several authors [23–26] in order to provide a more complete picture of the process. It is clear that Na^+/K^+-ATPase exists in two main conformations denoted E_1, with high Na^+-low K^+ affinity, and E_2, with high K^+-low Na^+ affinity. Interaction with ATP facilitates the transition from E_2 to E_1, allowing the binding of Na^+. Phosphorylation by ATP stimulates the transition from E_1 to E_2 and the outward transport of Na^+. K^+ binds the phosphoenzyme (E_2P), causing its dephosphorylation which, together with the subsequent interaction with ATP, is responsible for the inward transport of K^+ and the recovery of the initial state.

In this view, we propose that the acylphosphatase induced hydrolysis of the phosphoenzyme may occur in various steps (as indicated in Fig. 5), before and after the conformational changes needed for Na^+ transport, but at any rate, before the binding of $K^+(Rb^+)$. This could account for the increased but less efficient Na^+ transport, as evident from the diminuished Na^+/ATP ratio, and for the marked inhibition of $K^+(Rb^+)$ transport, since this cation binds only the phosphorylated form of Na^+,K^+ pump. In other words acylphosphatase would affect the coupling between ATP hydrolysis and cation transport altering the normal ordered reaction sequence and short-circuiting the system.

In conclusion, the present study provides the first evidence, at least to our knowledge, of a specific 'uncoupling' of RBC membrane Na^+,K^+ pump by an enzyme present in the same cells and used at concentrations within the normal range. Further investigations on the details of acylphosphatase action would be of interest to verify the possibility of a physiological role of this enzyme.

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