THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 266, No. 17, Issue of June 15, pp. 10867-10871, 1991
Printed in U.S.A.

Effects of Acylphosphatase on the Activity of Erythrocyte Membrane Ca^{2+} Pump*

(Received for publication, September 24, 1990)

Paolo Nassi‡, Chiara Nediani, Gianfranco Liguri, Niccolò Taddei, and Giampietro Ramponi

From the Dipartimento di Scienze Biochimiche, Università di Firenze, Viale G. Morgagni 50, 50134 Florence, Italy

Acylphosphatase, purified from human erythrocytes, actively hydrolyzes the acylphosphorylated intermediate of human red blood cell membrane Ca^{2+}-ATPase. This effect occurred with acylphosphatase amounts (up to 10 units/mg membrane protein) that fall within the physiological range. Furthermore, a very low K_m value, 3.41 ± 1.16 (S.E.) nM, suggests a high affinity in acylphosphatase for the phosphoenzyme intermediate, which is consistent with the small number of Ca^{2+}-ATPase units in human erythrocyte membrane. Acylphosphatase addition to red cell membranes resulted in a significant increase in the rate of ATP hydrolysis. Maximal stimulation (about 2-fold over basal) was obtained at 2 units/mg membrane protein, with a concomitant decrease in apparent K_m values for both Ca^{2+} and ATP. Conversely, similar amounts of acylphosphatase significantly decreased (by about 30%) the rate of Ca^{2+} transport into inside-out red cell membrane vesicles, albeit that reduced apparent K_m values for Ca^{2+} and ATP were also observed in this case. A stoichiometry of 2.04 Ca^{2+}/ATP hydrolyzed was calculated in the absence of acylphosphatase; in the presence of acylphosphatase optimal concentration, this ratio was reduced to 0.9. Acylphosphatase activity, rather than just protein, was essential for all the above effects.

Taken together these findings suggest that, because of its hydrolytic activity on the phosphoenzyme intermediate, acylphosphatase reduces the efficiency of the erythrocyte membrane Ca^{2+} pump. A possible mechanism for this effect is that the phosphoenzyme is hydrolyzed before its transport work can be accomplished.

Human red blood cell (RBC)[1] membrane has a Ca^{2+}-ATPase system that pumps Ca^{2+} ions out of the cell, coupling calcium transport to ATP hydrolysis. The combined effect of this active pumping mechanism and a slight Ca^{2+} leakage from outside to inside the cell enables the erythrocyte to maintain steady state intracellular concentrations below 10^{-6} M (1). Many studies have focused on the properties and the action mechanism of RBC membrane Ca^{2+}-ATPase, and accumulated evidence indicates that ATP hydrolysis proceeds through a series of elementary reactions that involve the Ca^{2+}-dependent formation of an acylphosphorylated intermediate (2). On the other hand, the efficiency of the calcium pump is still controversial since differing Ca^{2+}/ATP ratios have been reported (3), suggesting that the stoichiometry of this process may be 1 or 2 calcium ions transported per mol of ATP hydrolyzed. It is well known that human RBC Ca^{2+}-ATPase is activated by calmodulin and that this effect is associated with an increased rate in phosphorylated intermediate formation (2). Human RBCs, however, seem to contain other factors that stimulate Ca^{2+}-ATPase activity. A non-calmodulin activator in membrane preparations has been described by Mauldin and Roufogalis (4). Davis *et al.* (5) have reported evidence to suggest that hemolysate from human RBCs contains soluble Ca^{2+}-ATPase stimulators other than calmodulin. Wang *et al.* (6) have demonstrated that RBC membrane Ca^{2+}-ATPase is activated by calpain.

Acylphosphatase (EC 3.6.1.7) is a widespread cytosolic enzyme that catalyzes the hydrolysis of the carboxyl phosphate bond of acylphosphates, such as 3-phosphoglycerol phosphate (7), carbamoyl phosphate (8), and succinoyl phosphate (9). We have reported some structural and functional properties of acylphosphatase purified to homogeneity from the skeletal muscle of varying vertebrate species, including man (10–13). All these enzymes have similar kinetic properties and a highly conserved primary structure.

We have subsequently isolated from human RBCs an acylphosphatase isoenzyme (14) that exhibits similar substrate specificity but higher catalytic potency as compared with the muscular isoform.

We supposed that RBC acylphosphatase, on the basis of a potential hydrolytic effect on the acylphosphorylated intermediate, might represent an additional modulator of Ca^{2+}-ATPase that would affect both ATP hydrolysis and calcium transport across erythrocyte membrane. The present paper reports the studies that we conducted to examine this hypothesis.

MATERIALS AND METHODS

Enzymes, coenzymes and substrates were from Boehringer (Mannheim, Federal Republic of Germany (FRG)). Dextran (M_r = 147,000) was from Sigma Chemie (Grunwalder, FRG). All other chemicals were the best commercially available. [γ-^{32}P]ATP (3 Ci/mmol) and ^{45}Ca (35 mCi/mg calcium) were purchased from Radiochemical Centre, Amersham.

Acylphosphatase was purified from human erythrocytes according to Liguri *et al.* (14); the enzyme, isolated as a pure product, had a specific activity of 7,500 units/mg protein, using benzoyl phosphate as substrate (see below). Benzoyl phosphate was synthesized as per Camici *et al.* (15). All steps of the preparation of human erythrocyte membranes, inside-out red blood membrane vesicles, and RBC Ca^{2+}-

* This work was supported by grants from the Ministero della Pubblica Istruzione (Fondi 60%) and from the Consiglio Nazionale della Ricerche (Target Project on Biotechnology and Bioinstrumentation). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dipartimento di Scienze Biochimiche, Università di Firenze, Viale Morgagni 50, 50134 Florence, Italy. Fax: 0039-55-4222725.

[1] The abbreviations used are: RBC, red blood cell; Ca^{2+}-ATPase, calcium ion-dependent triphosphatase; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; EP, phosphoenzyme; IOVs, inside-out vesicles of red cell membranes; (Na^+ + K^+)-ATPase, sodium-potassium ion-dependent adenosine triphosphatase.

ATPase-phosphorylated intermediate were carried out at 4 °C.

Preparation of Human Erythrocyte Membranes—RBC membranes with low basal calcium ATPase activity were prepared using Thakar's method (16). Freshly drawn heparinized blood was centrifuged at 1,800 × *g* for 10 min to remove plasma and the leucocyte layer from erythrocytes. RBCs were washed twice in ice-cold saline at 1,800 × *g* for 10 min. The erythrocytes were then hemolyzed in 10 mM imidazole buffer, pH 7.4, 1 mM NaCl, 1 mM $MgCl_2$ at about 12% hematocrit and centrifuged at 48,000 × *g* for 20 min. The ghosts were washed three times in 5 mM phosphate buffer, pH 8.0, and twice in the hemolysis buffer. The hemoglobin-free membranes were finally resuspended in 10 mM Tris-HCl, pH 7.4, and stored at −80 °C.

Ca^{2+}-ATPase Assay—Ca^{2+}-ATPase activity in RBC membranes was determined by a coupled optical test at 340 nm and 37 °C, using a Perkin-Elmer λ 4B spectrophotometer. The medium contained 25 mM Tris-HCl, pH 7.4, 1 mM ATP, 5 mM $MgCl_2$, 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, 6 units of pyruvate kinase, 5.5 units of lactate dehydrogenase, 100 μg of membrane protein, and variable additions of acylphosphatase, as indicated in Table II.

Ca^{2+}-ATPase activity was calculated as the difference in ATP hydrolysis in the presence and the absence of Ca^{2+} (1 mM $CaCl_2$; 0.05 mM EGTA) and was expressed as nmol of ATP hydrolyzed/h/mg membrane protein.

Acylphosphatase Assay—Acylphosphatase activity was determined by a continuous optical method that was based on the difference in absorbance at 283 nm between benzoyl phosphate and benzoate at 25 °C (17). The unit of activity is defined as the amount of enzyme that liberates 1 μmol of benzoate/min.

Protein Determination—Membrane protein content was assayed with the biuret method of Beisenhertz *et al.* (18) using bovine serum albumin as a standard.

Preparation of RBC Membrane Ca^{2+}-ATPase-phosphorylated Intermediate—RBC membranes, prepared by the method of Garrahan *et al.* (19), were phosphorylated according to Luthra *et al.* (20). In a typical experiment, 1 ml of reaction mixture contained 0.0125 mM $MgCl_2$, 0.05 mM EGTA, 0.1 mM $CaCl_2$, 0.150 M Tris-HCl, pH 7.2, and 1.5 mg/ml membrane protein. The phosphorylation reaction, performed in ice, was initiated by the addition of [γ-^{32}P]ATP to give a final concentration of 10 μM ATP. The reaction was terminated by the addition of 5 ml of cold 6% trichloroacetic acid, 50 mM NaH_2PO_4, and 1 mM nonradioactive ATP. Membrane suspension was centrifuged at 30,000 × *g* for 10 min. Pellets were resuspended, washed twice in the above medium and three additional times in 0.150 M Tris-HCl, pH 7.2. An aliquot of the pellet was dissolved in 3% sodium dodecyl sulfate and checked for radioactivity in a model SL 30 Intertechnique counter; another aliquot was used for protein measurement. The level of phosphoenzyme was taken as the difference between the amount of ^{32}P incorporated into the membrane protein in a medium with Ca^{2+} and in a medium of identical composition except that Ca^{2+} was omitted.

Incubation of [^{32}P]Phosphoenzyme with Acylphosphatase—Labeled membranes were incubated in 0.150 M Tris-HCl, pH 7.2, at 37 °C in the presence of differing amounts of acylphosphatase as indicated in the figures; the reaction was terminated by adding final 10% trichloroacetic acid, and the suspensions were centrifuged at 30,000 × *g* for 20 min. Aliquots of the supernatants were used for measuring free ^{32}P radioactivity. Controls for spontaneous hydrolysis of the phosphorylated intermediate were incubated under the same conditions, except that acylphosphatase was omitted. As the phosphate release, measured at 2-min intervals for 10 min, was not linear, the initial rate of both spontaneous and acylphosphatase-induced hydrolysis was estimated by calculating the first derivative value at time zero of the curves that described the phosphate release as a function of time.

Ca^{2+} Influx Measurement into Inside-out Red Cell Membrane Vesicles (IOVs)—Inside-out red cell membrane vesicles were prepared by the method of Steck and Kant (21) and separated by layering the membrane suspension on 2–3-fold excess volume of 1.03 g/ml dextran barrier solution. The efficiency of the preparation of these vesicles (as judged by acetylcholinesterase accessibility) was 89%. 100 μg of IOVs were then incubated in 20 mM Tris-HCl, pH 7.4, 130 mM KCl, 2 mM $MgCl_2$, 0.5 mM ATP, 0.2 mM $CaCl_2$ (+0.1 μCi of ^{45}Ca) at 37 °C in both the presence and the absence of acylphosphatase.

Ca^{2+} influx into IOVs was measured at 4-min intervals for 16 min by a rapid filtration technique using a 0.6-μm pore Sartorius membrane filter (22).

ATP hydrolysis by IOVs was measured in the same incubation medium as for calcium uptake, using the above described procedure (see "Ca^{2+}-ATPase Assay").

Expression of the Results—Data presented under "Results" are the means of several determinations, the values of which yielded coefficients of variation that did not exceed (except where otherwise stated) 10%. Curves were drawn on the basis of the mean values, while kinetic constants were calculated from all the available values with a computer data analysis program, Enzfitter by Elsevier Biosoft, version 1.03. Statistical analysis was performed by Student's *t* test or, where indicated, by one-way analysis of variance.

RESULTS

Effect of Acylphosphatase on the Acylphosphorylated Intermediate of RBC Membrane Ca^{2+}-ATPase—Fig. 1 shows the time course of Ca^{2+}-ATPase phosphoenzyme (EP) formation. In our experimental conditions, maximum Ca^{2+}-dependent RBC membrane labeling was achieved after a 60-s incubation; at this time the quantity of bound phosphate was, on average, 2.03 pmol/mg membrane protein, a value which agrees with that reported by Luthra *et al.* (20), from whose method our procedure derives. Labeled membranes were incubated with varying amounts of acylphosphatase, from 2 to 10 units/mg membrane protein. These acylphosphatase/membrane protein ratios were chosen because they reflected, from a quantitative point of view, physiological condition; on the basis of acylphosphatase activity in human RBCs and of the protein content of human erythrocyte membrane, a ratio that ranges from 3 to 7 units/mg membrane protein may be calculated (23). As shown in Table I the release of phosphate, net of spontaneous hydrolysis, rose significantly with the increase in acylphosphatase/membrane protein ratio. In order to characterize kinetically the acylphosphatase effect, we incubated a fixed amount of this enzyme (5 units) with variable amounts of ^{32}P-labeled membranes and measured the initial velocity of dephosphorylation as a function of EP concentration. In these conditions we observed (Fig. 2) that the initial rate of enzymatic dephosphorylation rose with the increase in EP concentration along a hyperbolic curve with an apparent K_m = 3.41 ± 1.16 (S.E.) nM.

Effect of Acylphosphatase on RBC Membrane Ca^{2+}-ATPase Activity—To see if the above action on the acylphosphorylated intermediate resulted in modified functional properties in Ca^{2+}-ATPase, we investigated the effect of acylphosphatase addition on the rate of Ca^{2+}-dependent RBC membrane ATP hydrolysis. When added to the assay medium in concentrations that ranged from 0.5 to 10 units/mg membrane protein, acylphosphatase (which did not exhibit *per se* Ca^{2+}-ATPase activity) significantly increased the rate of RBC membranes ATP hydrolysis (Table II). The increase depended on the

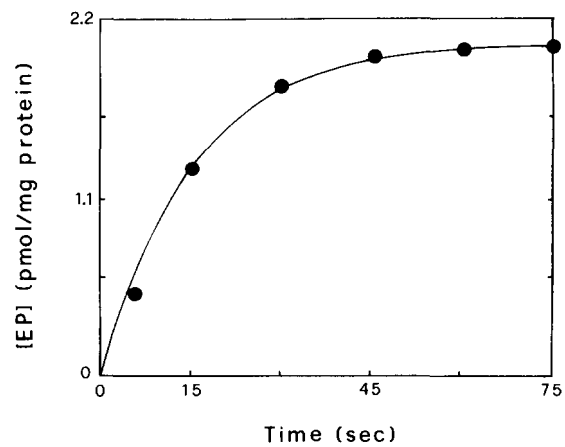

FIG. 1. **Time course of EP formation.** Membranes were phosphorylated and the level of phosphoenzyme was calculated as described under "Materials and Methods." Each *point* represents the mean value of four experiments.

TABLE I

Effect of different concentration of acylphosphatase on the Ca^{2+}-ATPase-phosphorylated intermediate from RBC membranes

Labeled membranes (1 mg of protein) were incubated in 0.150 M Tris-HCl, pH 7.2, at 37 °C with varying amounts of acylphosphatase from 2 to 10 units, in a final volume of 1 ml. The initial rate of phosphate release was calculated as described under "Materials and Methods." Results are means ± S.E. of five experiments performed on differing membrane preparations. All the changes in ^{32}P release induced by acylphosphatase addition were statistically significant ($p < 0.05$).

Sample	Phosphate bound	Phosphate released
	pmol/mg protein	*pmol/min*
Labeled membranes	1.81 ± 0.07	
Control for spontaneous hydrolysis		0.34 ± 0.06
Acylphosphatase-treated labeled membranes		
2 units/mg		0.43 ± 0.05
5 units/mg		0.61 ± 0.09
10 units/mg		0.80 ± 0.12

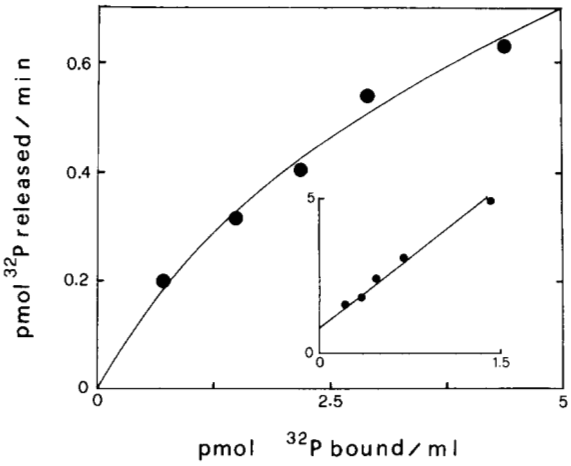

FIG. 2. **Initial rate of Ca^{2+}-ATPase intermediate dephosphorylation by acylphosphatase.** 5 units of acylphosphatase were incubated in 0.150 M Tris-HCl (pH 7.2 at 37 °C) with differing amounts of labeled membranes. EP concentration in the medium was expressed as pmol of ^{32}P bound/ml. The initial rate of dephosphorylation, net of spontaneous hydrolysis, was calculated as described under "Materials and Methods." Each *point* represents the mean value of four determinations. The *inset* shows the double-reciprocal plot of the data shown in the figure.

amount of acylphosphatase added and maximal stimulation (about 2-fold over basal value) was observed at 2 units/mg membrane protein. Higher acylphosphatase amounts did not produce further stimulation. However, comparing these data with those of Table I, it is noteworthy that the increase in Ca^{2+}-ATPase activity and the stimulation of ^{32}P release (both calculated as percentage of the values observed without acylphosphatase) were of the same order of magnitude.

To study the effect of acylphosphatase on the Ca^{2+} and ATP dependence of RBC membrane Ca^{2+}-ATPase activity, we measured the rate of ATP hydrolysis as a function of [Ca^{2+}] and [ATP], in the presence of optimal acylphosphatase concentration (2 units/mg membrane protein) and compared the observed values with those obtained without our enzyme. The results of these experiments are reported in Figs. 3 and 4. From the obtained curves, we may calculate that the presence of acylphosphatase, apart from increasing the maximum rate of ATP hydrolysis, shifts the apparent K_m value for Ca^{2+} from 19.66 ± 1.68 (S.E.) μM to 13.25 ± 1.38 (S.E.) μM, and that for ATP from 0.422 ± 0.039 (S.E.) mM to 0.306 ± 0.016 (S.E.) mM. These changes were statistically significant ($p < 0.05$).

Effect of Acylphosphatase on Ca^{2+} Influx into Inside-out RBC Membrane Vesicles—Active Ca^{2+} uptake in our IOV preparations was measured both in the presence and in the absence of differing amounts of acylphosphatase (Fig. 5). Measurements were taken at 4-min intervals over a 16-min period. When the system lacked acylphosphatase, Ca^{2+} influx proceeded linearly throughout the given period, at a rate which was calculated as 5.98 ± 0.27 (S.E.) nmol/min/mg IOV protein. When acylphosphatase was added to the system (2, 5, 7, or 10 units/mg IOV protein), we observed that the rate of Ca^{2+} influx decreased significantly and more markedly so with higher enzyme amounts. At 10 units/mg IOV protein, the rate of Ca^{2+} uptake was reduced to 4.09 ± 0.15 (S.E.) nmol/min/mg IOV protein, and the addition of larger amounts of acylphosphatase did not yield greater reduction values. When determining Ca^{2+} efflux from loaded IOVs we did not observe appreciable differences in Ca^{2+} loss with or without acylphosphatase; the enzyme, in addition, did not exhibit proteolytic activity. It seems therefore improbable that

TABLE II

Effect of exogenous acylphosphatase on erythrocyte membrane Ca^{2+}-ATPase activity

Results are means ± S.E. of six determinations performed on differing membrane preparations. Ca^{2+}-ATPase activity was measured as described under "Materials and Methods." The letter A represents Ca^{2+}-ATPase activity with acylphosphatase. ΔA indicates the change in Ca^{2+}-ATPase with acylphosphatase compared with the activity without this enzyme. Changes in Ca^{2+}-ATPase activity observed with increasing amounts of acylphosphatase were statistically significant ($p < 0.01$ by the one-way analysis of variance).

Acylphosphatase	Ca^{2+}-ATPase activity	
	A	ΔA
units/mg membrane protein	*nmol/h/mg membrane protein*	
0	109 ± 11	
0.5	134 ± 16	+25
1.0	145 ± 15	+36
1.5	157 ± 18	+48
2.0	213 ± 23	+104
5.0	208 ± 22	+99
10.0	201 ± 19	+92

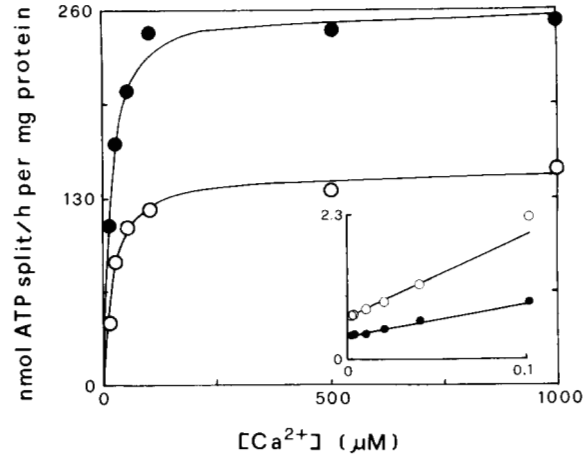

FIG. 3. **RBC membrane Ca^{2+}-ATPase activity as a function of Ca^{2+} concentration.** Except for varying Ca^{2+} concentrations, Ca^{2+}-ATPase activity was measured as described under "Materials and Methods," in the presence (●) and in the absence (○) of acylphosphatase (2 units/mg of membrane protein). Each *point* represents the mean value of four determinations. The *inset* shows the double-reciprocal plot of the data shown in the figure (y axis, $v^{-1} \times 10^2$).

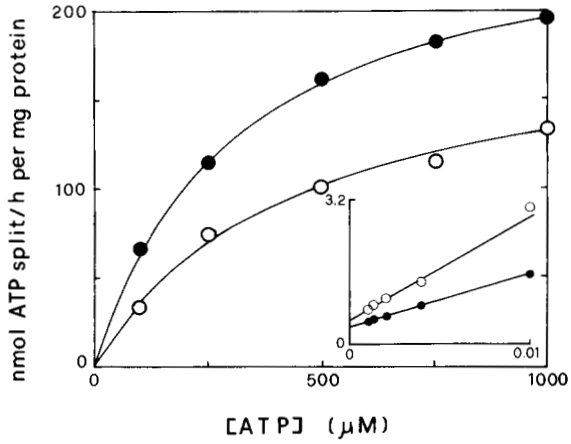

FIG. 4. **RBC membrane Ca^{2+}-ATPase activity as a function of ATP concentration.** Except for varying ATP concentrations, Ca^{2+}-ATPase activity was measured as described under "Materials and Methods," in the presence (●) and in the absence (○) of acylphosphatase (2 units/mg membrane protein). Each *point* represents the mean value of four determinations. The *inset* shows the double-reciprocal plot of the data shown in the figure (y axis, $v^{-1} \times 10^2$).

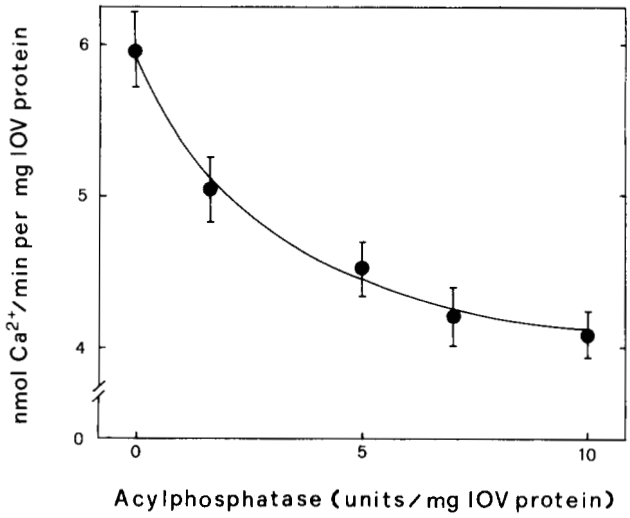

FIG. 5. **Effect of acylphosphatase on Ca^{2+} transport into RBC membrane inside-out vesicles.** Ca^{2+} uptake was assayed as described under "Materials and Methods" and was expressed as nmol/min/mg IOV protein. Acylphosphatase concentration is expressed in units/mg IOV protein. Each *point* represents the mean ± S.E. of four determinations. Changes observed with differing amounts of acylphosphatase were statistically significant ($p < 0.01$ by one-way analysis of variance).

the effect of acylphosphatase is simply due to an increased Ca^{2+} leakage from vesicles.

Under identical conditions to those used for Ca^{2+} uptake, we measured the rate of IOV ATP hydrolysis and found (mean values) 2.92 nmol/min/mg protein without acylphosphatase and 4.41 nmol/min/mg protein with acylphosphatase at the optimal concentration of 10 units/mg IOV protein. From these and the above data we may calculate that acylphosphatase, in these conditions, reduces on average the Ca^{2+}/ATP ratio from 2.04 to 0.91.

As for the dependence of active Ca^{2+} uptake by IOVs on the Ca^{2+} and ATP concentration, in the absence of acylphosphatase we calculated apparent K_m values of 30.54 ± 3.27 (S.E.) μM for Ca^{2+} and of 128.96 ± 9.27 (S.E.) μM for ATP; such findings agree favorably with other reports, in particular with those of Waisman *et al.* (24). In the same conditions, the addition of acylphosphatase (in this case also at the concentration of 10 units/mg IOV protein) resulted not only, as expected, in a diminished maximum rate of Ca^{2+} uptake, but also in a significant decrease ($p < 0.01$) in apparent K_m values which we calculated as 17.64 ± 2.16 (S.E.) μM and 86.52 ± 9.05 (S.E.) μM, respectively, for Ca^{2+} and ATP. These changes are of the same type and of the same order of magnitude as those observed on Ca^{2+}-ATPase activity.

Studies with Heat-inactivated Acylphosphatase—In order to establish if acylphosphatase activity was essential for the effects on the phosphoenzyme intermediate, Ca^{2+}-dependent ATP hydrolysis and calcium transport, similar experiments to those reported in Table I, Table II, and Fig. 5 were performed using heat-inactivated (2 h at 100 °C) enzyme. Acylphosphatase was added at the same concentrations that, with the active enzyme, gave the maximal effects. The results of these experiments (Table III) indicate that no significant change was induced by inactivated acylphosphatase.

DISCUSSION

We have here demonstrated that acylphosphatase actively hydrolyzes the phosphoenzyme intermediate of RBC membrane Ca^{2+}-ATPase. This was predictable (given the catalytic properties of our enzyme and the acylphosphate nature of the intermediate) all the more so, since the literature had already reported similar effect of acylphosphatase on the acylphosphorylated intermediates of other transport ATPases, such as brain ($Na^+ + K^+$)-ATPase (25) and sarcoplasmic reticulum Ca^{2+}-ATPase (26). However, the most remarkable feature of the action on the RBC membrane Ca^{2+}-ATPase phosphoenzyme is that, unlike the previous reports, the enzymatic hydrolysis occurs to a significant extent with acylphosphatase amounts that fall in the physiological range. Furthermore, the very low K_m value that we found for acylphosphatase hydrolysis suggests a high affinity in this enzyme for EP, which seems to be consistent with a small number of Ca^{2+}-ATPase units, these latter only accounting for about 0.1% of the total membrane proteins in human RBC membranes (2).

Another aspect that, in our opinion, should be emphasized is that the effect of acylphosphatase on EP was observed in RBC membranes not subjected to disruption, proteolysis, or other drastic treatments. This seems to provide a novel and interesting topographical implication suggesting that the catalytic site of Ca^{2+} pump might be accessible, also in intact RBC membranes, to an exogenous (nonparticulate, cytosolic) enzyme like acylphosphatase. In addition to this action on the acylphosphorylated intermediate, acylphosphatase affected Ca^{2+}-ATPase activity as regards both ATP hydrolysis and Ca^{2+} transport. However, the two effects were of opposite type. Our results, in fact, indicate that comparable amounts of acylphosphatase significantly increase the rate of Ca^{2+}-dependent ATP hydrolysis, while they decrease the rate of Ca^{2+} transport across RBC membranes. Maximal stimulation of ATP hydrolysis was observed at 2 acylphosphatase units/mg membrane protein and resulted in an increase of about 100% over basal values; maximal inhibition of Ca^{2+} transport was obtained with 10 units/mg membrane protein and resulted in a decrease of about 30% with respect to basal values. It is difficult to explain these differences as regards both the entity of the effects and the optimal concentration of our enzyme. It should be considered, however, that ATP hydrolysis was assayed in intact RBC membranes; these were prepared with a procedure that tended to obtain membranes possessing a low basal Ca^{2+}-ATPase activity and a high sensitivity to stimulating agents. Conversely, studies on Ca^{2+} transport were performed using internal-external vesicles from RBC membrane, fractionated on a dextran barrier. The

TABLE III

Effects of heat-inactivated acylphosphatase on RBC membrane Ca^{2+} pump

Results are means ± S.E. of five experiments performed on differing membrane preparations. All the changes induced by inactivated acylphosphatase were not statistically significant. A, experimental conditons were as reported in Table I; phosphate bound was 1.87 ± 0.06 pmol/mg protein; inactivated acylphosphatase was added at a concentration of 1.33 μg (corresponding to 10 units of the active enzyme) per mg membrane protein. B, experimental conditions as in Table II; inactivated acylphosphatase concentration: 0.26 μm/mg membrane protein. C, experimental conditions as in Fig. 5, inactivated acylphosphatase concentration: 1.33 μg/mg IOV protein.

	Without acylphosphatase	With inactivated acylphosphatase
A. Phosphate release (pmol/min)	0.42 ± 0.07	0.39 ± 0.06
B. Ca^{2+}-ATPase activity (nmol/h/mg membrane protein)	118 ± 10	122 ± 12
C. Ca^{2+} influx into inside-out membrane vesicles (nmol/min/mg IOV protein)	5.67 ± 0.32	5.31 ± 0.29

differences in membrane preparation and vesiculation might have yielded differing functional properties in Ca^{2+}-ATPase and/or changed interactions with our enzyme.

Another point to be discussed regards the Ca^{2+} and ATP dependence of RBC membranes in ATP splitting. Our findings, over the concentration examined, indicate substantially hyperbolic curves and linear double reciprocal plots, characterized by the above mentioned parameters. In contrast, several other investigators have reported biphasic curves and lower K_m values for both Ca^{2+} and ATP. In agreement with Scharff and Foder (27) we believe that this may be mainly ascribed to the procedure used for membrane preparation. Our membranes were prepared in the absence of calcium and were very low in residual calmodulin; it has been reported that the Ca^{2+}-dependent binding of calmodulin to RBC membrane shifts Ca^{2+}-ATPase to a state characterized by a positive cooperativity in calcium activation, higher affinity for Ca^{2+} and regulatory role of ATP (28).

In any case, from the results here reported, it appears that acylphosphatase, in its catalytically active form, has an "uncoupling" effect on the erythrocyte membrane Ca^{2+} pump. This was confirmed by the diminished Ca^{2+}/ATP ratio that we found in the presence of acylphosphatase when we measured, in the same medium, the rate of IOV Ca^{2+} uptake and the rate of ATP hydrolysis. As regards the mechanism of this effect, we propose that the acylphosphatase-induced hydrolysis of the phosphoenzyme intermediate, in competition with its own hydrolytic catalysis, occurs before it can be used for bringing about the conformational change that normally pumps calcium. In this view, the lowering of apparent K_m values for Ca^{2+} and ATP, both in calcium transport and ATP hydrolysis, is probably the result of a change in the contribution of various elementary steps to rate limitation of the entire reaction cycle, through which proceeds the operation of RBC membrane Ca^{2+} pump.

In conclusion, the present study does point out a novel mechanism that affects the efficiency of calcium transport across erythrocyte membrane. Further investigation about the details of acylphosphatase action and an hypothetic physiological role of this enzyme in erythrocyte calcium homeostasis would be of interest.

Acknowledgments—We thank Dr. Vincent C. Manganiello for valuable comments and Dr. Elena Marchetti for skillful assistance.

REFERENCES

1. Macintyre, J. D., and Green, J. W. (1978) *Biochim. Biophys. Acta* **510,** 373–377
2. Adamo, H. P., Rega, A. F., and Garrahan, P. J. (1988) *J. Biol. Chem.* **263,** 17548–17554
3. Roufogalis, B. D. (1979) *Can. J. Physiol. Pharmacol.* **57,** 1331–1349
4. Mauldin, D., and Roufogalis, B. D. (1980) *Biochem. J.* **187,** 507–513
5. Davis, F. B., Davis, P. J., and Blas, S. D. (1983) *J. Clin. Invest.* **71,** 579–586
6. Wang, K. K. W., Villalobo, A., and Roufogalis, B. D. (1988) *Arch. Biochem. Biophys.* **260,** 696–704
7. Ramponi, G., Treves, C., and Guerritore, A. (1967) *Experientia (Basel)* **23,** 1019–1020
8. Ramponi, G., Melani, F., and Guerritore, A. (1961) *G. Biochim.* **10,** 189–196
9. Berti, A., Stefani, M., Liguri, G., Camici, G., Manao, G., and Ramponi, G. (1977) *Ital. J. Biochem.* **26,** 377–378
10. Cappugi, G., Manao, G., Camici, G., and Ramponi, G. (1980) *J. Biol. Chem.* **255,** 6868–6874
11. Nassi, P., Liguri, G., Landi, N., Berti, A., Stefani, M., Pavolini, B., and Ramponi, G. (1985) *Biochem. Med.* **34,** 166–175
12. Manao, G., Camici, G., Cappugi, G., Stefani, M., Liguri, G., Berti, A., and Ramponi, G. (1985) *Arch. Biochem. Biophys.* **241,** 418–424
13. Manao, G., Cappugi, G., Modesti, A., Stefani, M., Marzocchini, R., Degli Innocenti, D., and Camici, G. (1988) *J. Protein Chem.* **7,** 417–426
14. Liguri, G., Camici, G., Manao, G., Cappugi, G., Nassi, P., Modesti, A., and Ramponi, G. (1986) *Biochemistry* **25,** 8089–8094
15. Camici, G., Manao, G., Cappugi, G., and Ramponi, G. (1976) *Experientia (Basel)* **32,** 535
16. Thakar, J. H. (1985) *Anal. Biochem.* **144,** 94–97
17. Ramponi, G., Treves, C., and Guerritore, A. (1966) *Experientia (Basel)* **22,** 705
18. Beisenhertz, G., Boltze, H. U., Bucher, Th., Czok, R., Garbade, K. H., Meyer-Arendt, G., and Pfleiderer, G. (1955) *Z. Naturforsch. Sect. B.* **8,** 555–557
19. Garrahan, P. J., Pouchan, M. I., and Rega, A. F. (1969) *J. Physiol. (Lond.)* **202,** 305–327
20. Luthra, M. G., Watts, R. P., Scherer, K. L., and Kim, H. D. (1980) *Biochim. Biophys. Acta* **633,** 299–304
21. Steck, T. L., and Kant, J. A. (1974) *Methods Enzymol.* **31,** 172–180
22. Sarkadi, B., Macintyre, J. D., and Gardos, G. (1978) *FEBS Lett.* **89,** 78–82
23. Liguri, G., Nassi, P., Degli Innocenti, D., Tremori, E., Nediani, C., Berti, A., and Ramponi, G. (1987) *Mech. Ageing Dev.* **39,** 59–67
24. Waisman, D. M., Gimble, J. M., Goodman, D. B. P., and Rasmussen, H. (1981) *J. Biol. Chem.* **256,** 409–414
25. Hokin, L. E., Sastry, P. S., Galsworthy, P. R., and Yoda, A. (1965) *Proc. Natl. Acad. Sci. U. S. A.* **54,** 177–184
26. Stefani, M., Liguri, G., Berti, A., Nassi, P., and Ramponi, G. (1981) *Arch. Biochem. Biophys.* **208,** 37–41
27. Scharff, O., and Foder, B. (1978) *Biochim. Biophys. Acta* **509,** 67–77
28. Muallem, S., and Karlish, S. J. D. (1981) *Biochim. Biophys. Acta* **647,** 73–86