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Ca^{2+}-STIMULATED MEMBRANE PHOSPHORYLATION AND ATPase ACTIVITY OF THE HUMAN ERYTHROCYTE

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SUMMARY

1. Human erythrocyte membranes were preincubated with ethyleneglycolbis-(β-aminoethyl)-*N,N'* tetraacetate (EGTA) and subsequently labelled for short periods with micromolar concentrations of [8-^{3}H, γ-^{32}P]ATP. Under these conditions, and at temperatures $\leqq$ 22 °C, both ATP hydrolysis and membrane phosphorylation were stimulated by Ca^{2+}.

2. The properties of the Ca^{2+}-stimulated ATP hydrolysis and associated phosphorylation of a 150 000 molecular weight protein component, previously described (Knauf, P. A., Proverbio, F. and Hoffman, J. F. (1974) J. Gen. Physiol. 63, 324–336), have been studied. The behavior of the phosphorylated component, $E_{Ca}P$, has properties consistent with its role as a phosphorylated intermediate of Ca^{2+}-ATPase activity, including: (1) similar dependence of the steady-state level of $E_{Ca}P$ and Ca^{2+}-ATPase on ATP concentration; (2) rapid turnover apparent upon the addition of excess non-radioactive ATP; and (3) good correlation between the steady-state levels of Ca^{2+}-dependent phosphorylation and Ca^{2+}-ATPase activity in separate preparations possessing variable specific activity. Addition of excess EGTA to $E_{Ca}P$ caused only partial dephosphorylation. Sensitivity of Ca^{2+}-stimulated ATP hydrolysis and associated phosphorylation to micromolar concentrations of Ca^{2+} implicates this activity in the "high-affinity" Ca^{2+}-pump system of the human erythrocyte (Schatzmann, H. J. (1973) J. Physiol. London 235, 551–569).

INTRODUCTION

Studies by Schatzmann and others have shown that the intracellular level of free Ca^{2+} is maintained at a low level in the human erythrocyte by an energy-dependent pump located in the cell membrane [1–3]. There is good evidence implicating ATP as the direct energy source for the Ca^{2+} pump and suggesting that this transport system is manifested in the isolated plasma membrane as a Mg^{2+}-dependent Ca^{2+}-activated ATPase activity (Ca^{2+}-ATPase) [1, 4, 5]. Schatzmann [3], working with

Abbreviation: EGTA, ethyleneglycolbis-(β-aminoethyl)-*N,N'*-tetraacetate.

resealed human red cells loaded with a Ca-ethyleneglycolbis-(β-aminoethyl)-*N,N'*-tetraacetate (EGTA) buffer, found that the intracellular free Ca^{2+} concentration for half-saturation of the Ca^{2+} pump is $4 \cdot 10^{-6}$ M which is similar to the dissociation constant of the Ca^{2+}-ATPase. The stoichiometry between Ca^{2+} transported and ATP hydrolyzed appears to be 1 : 1 rather than 2 : 1 as in the sarcoplasmic reticulum [6].

In a recent study, Knauf et al. [7] demonstrated that Ca^{2+} can stimulate the phosphorylation of protein in erythrocyte membranes following incubation with [γ-^{32}P]ATP, Ca^{2+} and Mg^{2+}. They also showed this phosphoprotein to be distinct from that implicated as the intermediate in the Na^+-ATPase system [8, 9]. By analogy with the known mechanism of plasma-membrane Na^+-ATPase and Ca^{2+}-ATPase in the sarcoplasmic reticulum [10], this phosphoprotein may be an intermediate of erythrocyte membrane Ca^{2+}-ATPase. However, further investigation is needed to relate Ca^{2+}-ATPase activity to the ATP-dependent Ca^{2+} pump and to gain insight into the mechanism of such a pump-ATPase system. This study was undertaken to determine the relationship of the Ca^{2+}-phosphoprotein in red cell membrane to Ca^{2+}-ATPase, particularly that component sensitive to low Ca^{2+} (see ref. 3), and to elucidate further some of the properties of this phosphoprotein. A preliminary report has been presented [11].

MATERIALS AND METHODS

Erythrocyte membranes were prepared by osmotic lysis of fresh saline-washed human erythrocytes. After centrifugation, the post-hemolysate residue was washed with solutions containing EDTA as described previously [12]. The residue was made up to one-half the original packed cell volume of the washed cells and stored at 4 °C. The preparation obtained contained approximately 4 mg/ml protein, and was used within 6 days of preparation. The membrane preparation was frozen and thawed twice before use, to ensure complete permeability of substrates.

During the course of experiments on erythrocyte membrane phosphorylation a significant degree of ^{3}H-labelled nucleotide binding was observed. In view of this, it was necessary to measure and correct for this binding using [8-^{3}H, γ-^{32}P]ATP, as described previously [13].

The preparation of [γ-^{32}P]ATP and the procedures for (a) labelling the membranes with [8-^{3}H, γ-^{32}P]ATP and (b) measuring ATPase activity as $^{32}P_i$ released from [γ-^{32}P]ATP have been described previously [8, 12]. When Ca^{2+} was added to the incubation medium, EGTA in a final concentration of 0.1 mM was present, both in the membrane preparation and in the incubation medium. The free Ca^{2+} concentration was then determined by the association constant for the interaction of EGTA with Ca^{2+} at pH 7.4 [14]. When ouabain was used, it was preincubated at a concentration of 1 mM with the erythrocyte membranes for at least 20 min at 37 °C, prior to equilibration at the reaction temperature. Sodium dodecylsulfate-polyacrylamide gel electrophoresis on 5 % gels was carried out according to the method of Lenard [15]. 6-cm gels were used and the gels run at 10 mA/tube until the marker dye was 0.5–1.0 cm from the bottom. 1- or 2-mm sections of the gel were minced by means of a gel slicer (Gilson Co.) and counted in a liquid scintillation counter using non-diluted Aquasol (New England Nuclear) as the counting medium. The gels were calibrated

with the following molecular weight markers: γ-globulin, bovine serum albumin, trypsin and pepsin. An internal marker consisting of membranes prepared from ^{125}I-labelled red cells [16] was run with each gel.

Tris and ATP were purchased from Sigma, $^{32}P_i$ was obtained from New England Nuclear and [8-^{3}H]ATP was obtained from Schwarz Bio-Research.

RESULTS

In order to detect and characterize a Ca^{2+}-phosphoprotein associated with red cell-membrane Ca^{2+}-ATPase, it was necessary to find conditions in which the behavior of both the phosphoprotein and ATP hydrolytic activity could be studied concomitantly. In the first place, it was necessary to use low concentrations of ATP to minimize non-specific membrane phosphorylation, as described previously in studies of Na^+-ATPase [12] and secondly, to use low Ca^{2+} concentrations, since Ca^{2+}-ATPase involved in Ca^{2+} pumping should, presumably, have a low K_m for Ca^{2+} (see ref. 3). Accordingly, the effect of Ca^{2+} (20 μM free Ca^{2+}) on ATP hydrolysis was measured with 2 μM ATP at 37 °C.

As shown in Fig. 1, either omission of EGTA or addition of Ca^{2+} increased ATP hydrolysis above that observed with EGTA alone. In contrast, the Na^+-stimulated component of ATP hydrolysis was maximal in the absence of added Ca^{2+}

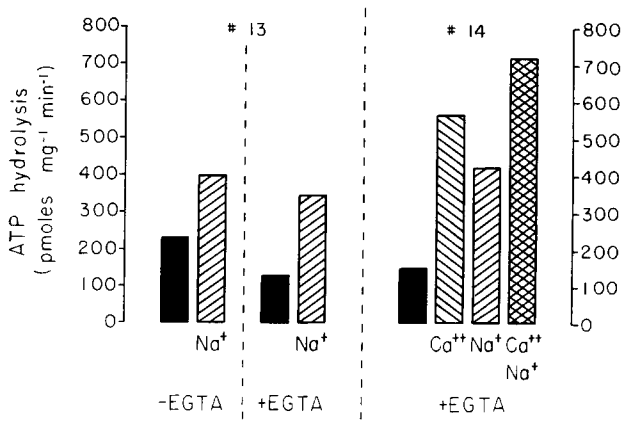

Fig. 1. Effect of Ca^{2+} on ATP hydrolysis. Erythrocyte membranes were preincubated for 10 min at 37 °C in the presence or absence of 0.1 mM Tris/EGTA, pH 7.4. Assays were carried out for 2 min at 37 °C in a final volume of 0.25 ml containing 0.1 ml membranes (2.0 mg protein/ml), 0.002 mM [γ-^{32}P]ATP ($90 \cdot 10^3$ cpm per 0.25 ml volume), 0.012 mM $MgCl_2$, 40 mM Tris · HCl, pH 7.4, and either 0.12 mM $CaCl_2$ (0.02 mM free Ca^{2+}) or 50 mM NaCl as indicated. The reaction was terminated and the $^{32}P_i$ released was measured as described previously [8, 12]. ATPase activity is expressed as $pmol \cdot mg^{-1} \cdot min^{-1}$.

(EGTA present) in agreement with previous studies showing that low Ca^{2+} levels decrease the affinity of Na^+-ATPase for Na^+ [17]. In the presence of Na^+ and Ca^{2+} together, the amount of ATP hydrolysis was greater than that observed in the presence of Na^+ or Ca^{2+} alone. A Ca^{2+}-phosphoprotein, $E_{Ca}P$, could also be detected under

these conditions, but only if the temperature was reduced to $\leqq$ 22 °C (see below and Figs 6 and 7).

Relationship of Ca^{2+}-ATPase to Ca^{2+}-stimulated membrane phosphorylation

In studies of the Na^+ pump and the Na^+-ATPase system, specific pump inhibitors, in particular the cardiac glycosides, have provided an invaluable means of delineating the relationship between the two activities. In this respect, the Ca^{2+}-transport system is less amenable to a similar comparison. It has been possible, however, to find conditions in which both the steady-state level of Ca^{2+}-phosphoprotein and Ca^{2+}-ATPase are affected similarly and thus to infer that the two are related.

One criterion for their relationship is the observation that, whereas both activities vary from preparation to preparation, the magnitude and direction of variation were similar for both. This is shown in Fig. 2 for 12 membrane preparations, each

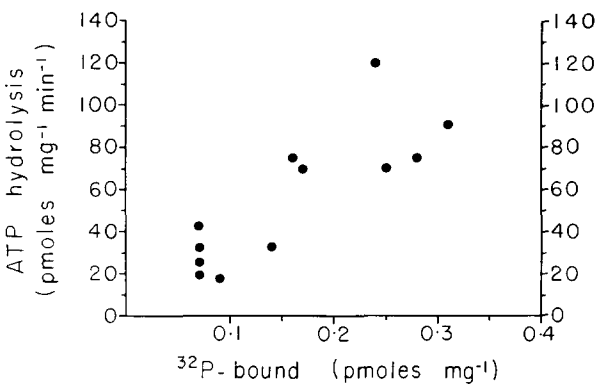

Fig. 2. Relationship of Ca^{2+}-stimulated ATPase activity to Ca^{2+}-stimulated phosphorylation. Erythrocyte membranes were incubated at 37 °C for 10 min in the presence of 0.1 mM Tris/EGTA, pH 7.4, and then at 22 °C in a final volume of 0.1 ml containing 0.07 ml membranes (4.0 mg protein/ml for phosphorylation and 2.0 mg/ml for hydrolysis measurements), 40 mM Tris · HCl, pH 7.4, 0.002 mM [8-^{3}H, γ-^{32}P]ATP (500 · 10^3 cpm ^{3}H or ^{32}P per 0.1 ml final volume), 0.012 mM $MgCl_2$, in the (a) absence or (b) presence of 0.12 mM $CaCl_2$ (0.02 mM free Ca^{2+}). The reaction was terminated and the ^{32}P bound and ATPase activity were measured as described previously [8, 12]. The values plotted are the differences (b−a). Phosphorylation, expressed as ^{32}P binding (pmol · mg^{-1}), following subtraction of [8-^{3}H]ATP bound, and ATPase activity (pmol · mg^{-1} · min^{-1}) were measured in separate reaction tubes under identical conditions. Reaction time was 2 min for ATPase activity and 10 s for the measurement of phosphorylation. ATPase and ^{32}P binding refer to Ca^{2+}-stimulated components of hydrolysis and phosphorylation, after subtraction of values measured without $CaCl_2$ added.

from a different sample of blood. The free Ca^{2+} concentration in this study was 20 μM. As shown, the Ca^{2+}-ATPase activity was associated and correlated well with the steady-state level of Ca^{2+}-phosphoprotein ($r = 0.91$).

When the ATP concentration was varied within a 50-fold range, Ca^{2+}-ATPase and the level of Ca^{2+}-phosphoprotein showed a similar dependence on the ATP concentration, i.e. the ratio of the two remained relatively constant. This is shown for a representative experiment (Fig. 3) in which the ATP was varied from 0.2 to 10 μM.

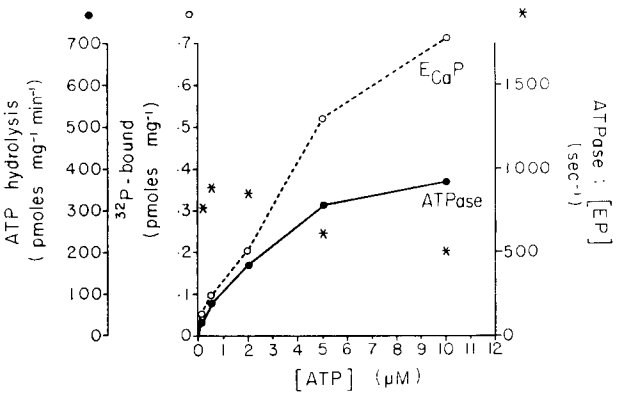

Fig. 3. Effect of ATP concentration on Ca^{2+}-stimulated phosphorylation and ATP hydrolysis Assays were carried out as described in Fig. 2, in the presence of 0.025 mM $MgCl_2$ and varying amounts of ATP (0.2 to 10 μM [8-^{3}H, γ-^{32}P]ATP ($300 \cdot 10^3$–$1500 \cdot 10^3$ cpm ^{3}H or ^{32}P per 0.1 ml volume). The reaction time was 10 s at 22 °C for the measurement of phosphorylation and 10 s at 0.2 and 0.5 μM ATP, 1 min at 2.0 μM, 3 min at 5.0 μM ATP and 5 min at 10 μM ATP for the measurement of ATPase activity. The reaction was terminated as described previously [8, 12]. The symbol * represents the ratio of ATPase activity to $E_{Ca}P$ at the different ATP concentrations.

Another property of the Ca^{2+}-phosphoprotein which is consistent with its participation in Ca^{2+}-ATPase activity was its rapid turnover. This was evident upon addition of excess non-radioactive ATP or addition of EGTA, as described below (see Fig. 8).

Properties of the Ca^{2+}-phosphoprotein

Effect of Ca^{2+}. Fig. 4 illustrates the effect of varying the Ca^{2+} concentration on ATPase activity and membrane phosphorylation. Ca^{2+}-ATPase reached a maxi-

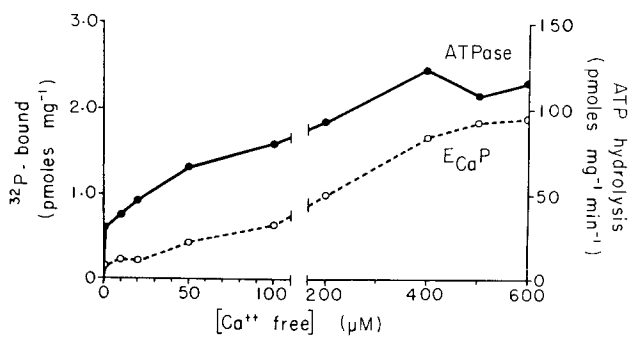

Fig. 4. Effect of Ca^{2+} on ATP hydrolysis and membrane phosphorylation. Membranes (4.0 mg protein/ml for phosphorylation and 1.9 mg/ml for hydrolysis measurements) were initially incubated at 37 °C for 10 min in the presence of 0.1 mM Tris/EGTA, pH 7.4, and then equilibrated for 10 min at 15 °C. Assays were carried out at 15 °C without and with varying amounts of added $CaCl_2$ (0.1–0.7 mM) as described in Fig. 2. Reaction time was 1.5 min for measurement of ATPase activity and 15 s for measurement of phosphorylation. The concentration of free Ca^{2+} was calculated as described by Caldwell [14]. ATPase and $E_{Ca}P$ refer to Ca^{2+}-stimulated components of hydrolysis and phosphorylation, after subtraction of the values measured without $CaCl_2$ added.

mum at Ca^{2+} concentrations lower than that which saturated Ca^{2+}-stimulated phosphorylation. Thus the turnover (ratio of hydrolysis to steady-state level of Ca^{2+}-phosphoprotein ($E_{Ca}P$)) decreased as Ca^{2+} increased. The possibility that there is a component of phosphorylation at higher Ca^{2+} in addition to and distinct from that observed at low Ca^{2+} concentrations was then tested. It was found that phosphorylation in the presence of high Ca^{2+} concentrations had the same degree of hydroxylamine sensitivity and showed the same electrophoretic mobility on sodium dodecylsulfate-polyacrylamide gels as phosphorylation in the presence of low Ca^{2+} concentrations (not shown). Similar findings were reported earlier by Knauf et al. [7]. In five experiments it was found that with Ca^{2+} concentrations in the range 2–100 μM, the Ca^{2+} required for half maximal activity of Ca^{2+}-ATPase ranged from 10^{-5} to 10^{-6} M, in close agreement with results of others [18, 3] and with the K_m reported for the ATP-dependent Ca^{2+} pump of human red cells [3]. At higher Ca^{2+} concentrations, deviation from Michaelis-Menten kinetics suggested a low-affinity Ca^{2+}-ATPase as well [3].

Effect of Mg^{2+}. As the Mg^{2+} concentration of the reaction medium was increased (from 12 to 500 μM) both the Mg^{2+}-dependent and ($Mg^{2+}+Ca^{2+}$)-dependent components of phosphorylation increased similarly (Fig. 5). Thus the

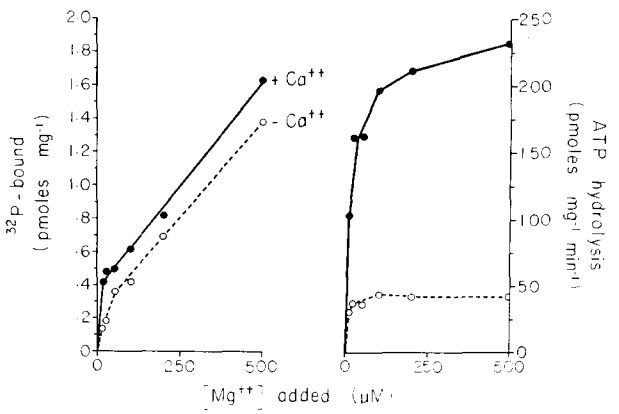

Fig. 5. Effect of Mg^{2+} concentration on Ca^{2+}-stimulated phosphorylation and ATP hydrolysis. Erythrocyte membranes were initially incubated for 10 min at 37 °C in the presence of 0.1 mM Tris/EGTA, pH 7.4, and then equilibrated for 10 min at 22 °C. Assays were carried out as described in Fig. 2 without (○- - -○) and with (●—●) 0.12 mM $CaCl_2$ (0.02 mM free Ca^{2+}) and with amounts of $MgCl_2$ ranging from 0.012 to 0.500 mM. Reaction time was 5 min for measurement of ATPase activity and 15 s for measurement of phosphorylation.

Ca^{2+}-stimulated phosphorylation ($Mg^{2+}+Ca^{2+}$)-component minus Mg^{2+}-component) remained constant throughout. In contrast Ca^{2+}-ATPase activity increased with increasing Mg^{2+} concentration. As a result, the turnover of $E_{Ca}P$, i.e. the ratio of hydrolysis to the level of $E_{Ca}P$, increased with increasing Mg^{2+} concentration.

Effect of temperature. As the temperature of the reaction was decreased, Ca^{2+}-ATPase activity was found to be reduced, e.g. in a typical preparation, from 34

pmol/mg protein per min at 22 °C to 9 pmol/mg protein per min at 10 °C. However, as shown in Fig. 6, lowering the temperature increased the steady-state level of $E_{Ca}P$. In contrast, Na^+-stimulated phosphorylation ($E_{Na}P$) was somewhat decreased so

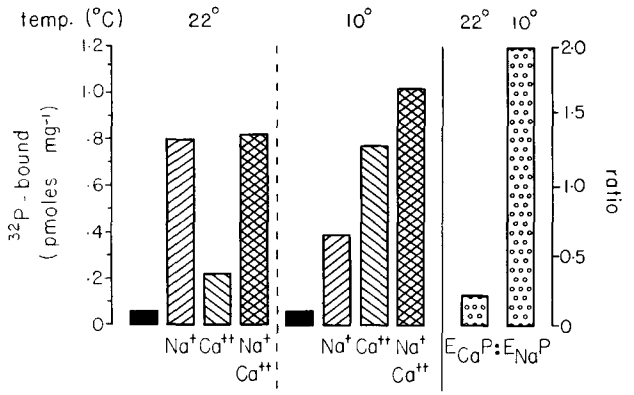

Fig. 6. Effect of temperature on $E_{Ca}P$ and $E_{Na}P$. Erythrocyte membranes were initially incubated at 37 °C for 10 min in the presence of 0.1 mM Tris/EGTA, pH 7.4, and then equilibrated for 10 min at the reaction temperature. Assays were carried out for 15 s at either 22 or 10 °C in a final volume of 0.1 ml containing 0.07 ml membranes (4 mg protein/ml) and [8-^{3}H, γ-^{32}P]ATP, and $MgCl_2$ as described in Fig. 2. 50 mM NaCl and 0.12 mM $CaCl_2$ (0.02 mM free Ca^{2+}) were included as indicated.

that the relative ratio $E_{Ca}P : E_{Na}P$ increased 8-fold with this temperature change. A more detailed illustration of this effect of temperature on Ca^{2+}-stimulated phosphorylation and ATP hydrolysis is shown in Fig. 7 at varying Ca^{2+} concentrations (20, 99 and 500 μM free Ca^{2+}). Whereas Ca^{2+}-ATPase increased as the temperature was increased from 0 to 22 °C, the level of Ca^{2+}-stimulated phosphorylation decreased steadily at temperatures above 8 °C.

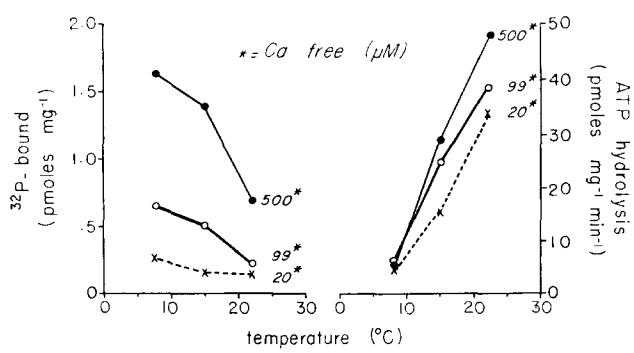

Fig. 7. Effect of temperature on Ca^{2+}-stimulated phosphorylation and ATP hydrolysis. Erythrocyte membranes were assayed as described in Fig. 6. Varying amounts of $CaCl_2$ were added to give the indicated concentrations of free Ca^{2+}. The reaction times were 15 s for measurement of ^{32}P binding, 40, 25 and 4 min for the measurement of $^{32}P_i$ released at 8, 15 and 22 °C, respectively.

Turnover of $E_{Ca}P$. Ca^{2+}-stimulated phosphorylation undergoes rapid dephosphorylation in the presence of EDTA and EGTA. Fig. 8 shows a time-course of the effect of EGTA (2 mM) on Ca^{2+}-stimulated phosphorylation; this is compared with that observed following the addition of a large excess of non-radioactive ATP (2 mM).

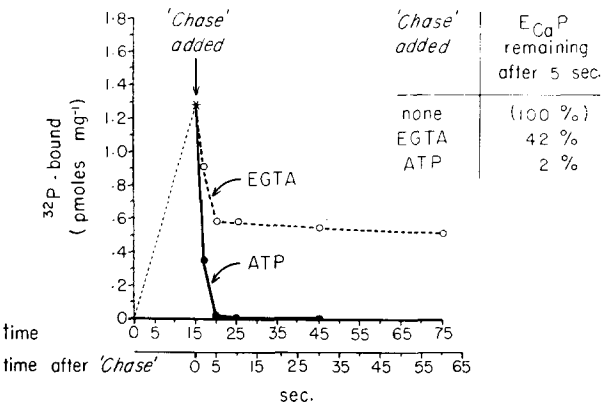

Fig. 8. Turnover of $E_{Ca}P$. Membranes were labelled as described in Fig. 2, for 15 s at 10 °C. At the end of this period either 2 mM EGTA or 1 mM Tris-ATP was added and the reaction continued and then terminated, at the indicated times.

Membranes were labelled with Ca^{2+} present, as previously described; at 15 s either EGTA or ATP was added to these samples and the reaction continued and then stopped at the indicated times. Following the addition of ATP, Ca^{2+}-stimulated ^{32}P-binding was decreased rapidly and completely within 5 s. The rate of dephosphorylation in the presence of EGTA was rapid at first, reducing the level of Ca^{2+}-stimulated phosphorylation to 50 % of the original levels within 5 s. Following this rapid dephosphorylation, there was little further decrease in Ca^{2+}-stimulated ^{32}P-binding either with time or with higher concentrations of EGTA or EDTA (not shown). Attempts were made to determine if the Ca^{2+}-stimulated phosphorylation consisted of two components, one that is rapidly dephosphorylated by EGTA or EDTA and one that is insensitive to this treatment; distinct components of Ca^{2+}-stimulated phosphorylation could not be separated by a difference in electrophoretic behavior or by a different degree of sensitivity to hydroxylamine. Treatment with detergent (20 μl/mg Triton X-100) as a means to further disrupt the membranes and increase accessibility of Ca^{2+} to the chelators did not increase the amount of dephosphorylation of $E_{Ca}P$.

Physicochemical properties of the Ca^{2+}-phosphoprotein

Table I indicates that the Ca^{2+}-stimulated phosphorylation was sensitive to hydroxylamine treatment to the same extent as the Na^+-stimulated phosphorylation of red cell membranes. This sensitivity to hydroxylamine suggests that $E_{Ca}P$ is an acyl phosphate intermediate [19]. These results confirm those of Knauf et al. [7] who tested the phosphorylation at 0 °C in the presence of 0.5 mM Ca^{2+}.

Polyacrylamide gel electrophoresis of red cell membranes labelled in the pres-

TABLE I

HYDROXYLAMINE SENSITIVITY OF Na^+- AND Ca^{2+}-STIMULATED PHOSPHORYLATION

Erythrocyte membranes (4.0 mg protein/ml) were initially incubated for 10 min at 37 °C in the presence of 0.1 mM Tris/EGTA (pH 7.4) and then for 10 min at 22 °C. Assays were carried out at 22 °C (10 s) in a final volume of 0.5 ml containing 0.35 ml membranes, 0.002 mM [8-^{3}H, γ-^{32}P]ATP ($450 \cdot 10^3$ cpm per 0.5 ml volume), 0.012 mM $MgCl_2$, 40 mM Tris · HCl, pH 7.4, and 0.1 mM EGTA and 0.12 mM $CaCl_2$ (0.02 mM free Ca^{2+}) or 50 mM NaCl as indicated. The reaction was terminated with 5 % trichloroacetic acid containing ATP and P_i as previously described [8, 12]. The membranes were washed five times in the same trichloroacetic acid solution then once in 0.02 M HCl and then exposed to 0.6 M hydroxylamine (NH_2OH) in 0.8 M sodium acetate at pH 5.2 for 10 min at 22 °C. ^{32}P bound, expressed as pmol · mg^{-1} protein, was measured as previously described [12]. In "control" membranes, exposed to 0.6 M NaCl under identical conditions, the amounts of ^{32}P released, 0.033 pmol · mg^{-1} (Ca^{2+}-stimulated phosphorylation) and 0.062 pmol · mg^{-1} (Na^+-stimulated phosphorylation), were subtracted from the amounts liberated in the presence of hydroxylamine.

Parameter measured	^{32}P bound (pmol · mg^{-1})	^{32}P released by NH_2OH (pmol · mg^{-1})
Ca^{2+}-stimulated phosphorylation	0.143	0.130
Na^+-stimulated phosphorylation	0.529	0.510

ence of Ca^{2+} or Na^+ revealed, in each case, a single major peak of radioactivity, the Ca^{2+}-stimulated component having a somewhat lower mobility than the Na^+ component. These findings confirm similar earlier findings of Knauf et al. [7]. Fig. 9a illustrates that when membranes were labelled with both Na^+ and Ca^{2+} present and

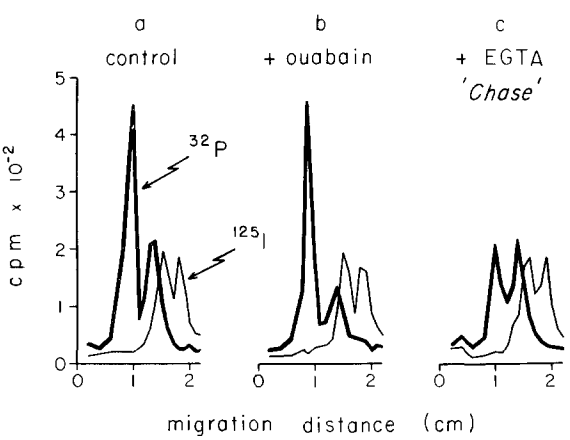

Fig. 9. Sodium dodecylsulfate gel electrophoresis of ^{32}P-labelled membranes. (a) membranes were labelled for 15 s at 10 °C as described in Fig. 2, in the presence of 50 mM NaCl and 0.12 mM $CaCl_2$ (0.02 mM free Ca^{2+}). The trichloroacetic acid-precipitated membranes were washed as previously described [8, 12] except that the final wash medium was 5 mM sodium phosphate, pH 2.4. The membrane pellets were resuspended in 0.06 ml of medium containing a 4 : 1 ratio of 5 mM sodium phosphate, pH 2.4, and 10 % sodium dodecylsulfate and then mixed with membranes from red blood cells, labelled with ^{125}I (ref. 16), 40 mM dithiothreitol, 0.002 % bromophenol blue and 20 % glycerin in a final total volume of 0.1 ml. In part b the identical conditions were used except that the membranes were preincubated with 1 mM ouabain for 30 min at 37 °C and then for 10 min at 10 °C prior to labelling. In part c, membranes were labelled as in a, above and at 15 s following the start of the reaction, 2 mM EGTA was added and the reaction continued for an additional 5 s.

then electrophoresed, two distinct peaks of radioactivity were observed: the peak with higher mobility was found to be ouabain-sensitive (Fig. 9b), the R_F of this peak corresponding to a molecular weight of approx. 110 000. This peak has been previously described as being membrane phosphorylation associated with the Na^+-dependent phosphorylated intermediate of the Mg^{2+}-dependent, $(Na^+ + K^+)$-activated ATPase (ATP phosphohydrolase, EC 3.6.1.3) [9]. The peak with the lower mobility, corresponding to a molecular weight of approx. 150 000, was found to be ouabain-insensitive but was partially sensitive to rapid dephosphorylation by EGTA (Fig. 9c). This peak of radioactivity thus corresponds to Ca^{2+}-stimulated membrane phosphorylation associated with Ca^{2+}-ATPase. A molecular weight of 150 000 for $E_{Ca}P$ was obtained when the membranes were electrophoresed on either 5 % gels (Fig. 9) or 3 % gels (not shown).

In order to further characterize these phosphoproteins in terms of their molecular weights relative to other components, the ^{32}P-labelled membranes were mixed with membranes prepared from ^{125}I-labelled red cells. The ^{125}I-labelled proteins are those exposed on the outer surface of the red cell membrane, the peak with lower mobility corresponding to the 100 000 molecular weight component(s) which span(s) the membrane [16]. As shown, both the Ca^{2+}-phosphoprotein, and the Na^+-phosphoprotein are distinct from the ^{125}I-peaks of radioactivity.

DISCUSSION

These studies have shown that a considerable component of ATP hydrolytic activity of the human erythrocyte membrane is Ca^{2+}-sensitive. Associated with this Ca^{2+}-ATPase is Ca^{2+}-stimulated membrane phosphorylation referred to as $E_{Ca}P$. Participation of $E_{Ca}P$ in Ca^{2+}-ATPase is suggested by (a) rapid turnover upon the addition of excess non-radioactive ATP and rapid disappearance following addition of EGTA or EDTA, (b) close correlation with Ca^{2+}-ATPase activity measured in separate preparations possessing variable specific activity and (c) similar dependence of the steady-state level of $E_{Ca}P$ and Ca^{2+}-ATPase on ATP concentration.

This study confirms previous findings of Knauf et al. [7] which suggest that the Ca^{2+}-stimulated phosphorylation of a 150 000 molecular weight component of the red cell membrane is an intermediate of Ca^{2+}-ATPase. Most of their experiments were carried out at relatively high Ca^{2+} concentrations (0.5 mM), the present ones, at relatively low Ca^{2+} concentrations, without apparent discrepancies noted in the chemical behavior of the Ca^{2+}-phosphoprotein (i. e. molecular weight and sensitivity to hydroxylaminolysis). Our data show, however, that the turnover of $E_{Ca}P$ is slower at higher Ca^{2+} than at lower Ca^{2+} levels. Since Mg^{2+} has the opposite effect, i.e. $E_{Ca}P$ turnover is increased by Mg^{2+}, this may reflect Ca^{2+}-Mg^{2+} competition, presumably after initial phosphorylation. That the system may be relatively complex and involve Mg^{2+}- and Ca^{2+}-sensitive equilibria between two or more forms of phosphoenzyme is suggested by the observation that excess EGTA or EDTA resulted in only a partial disappearance of $E_{Ca}P$. It remains possible, however, that some Ca^{2+} becomes firmly bound to the enzyme and cannot be released following EGTA treatment. The "non-chaseable" component of Ca^{2+}-stimulated phosphorylation could not be distinguished from the "rapidly-chased" component either by a difference in electrophoretic behavior or degree of hydroxylamine sensitivity.

The effect of temperature on the Na^+-ATPase and Ca^{2+}-ATPase systems further differentiates the two enzyme systems; decreasing the temperature increased $E_{Ca}P$ and decreased $E_{Na}P$ while at decreased temperature Ca^{2+}-ATPase activity was reduced.

The sensitivity of this Ca^{2+}-stimulated ATP hydrolysis and phosphorylation to micromolar concentrations of Ca^{2+} implicates this activity as part of the high-affinity Ca^{2+} pump system of the human erythrocyte [3].

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