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EFFECTS OF DIVALENT METAL IONS ON THE CALCIUM PUMP AND MEMBRANE PHOSPHORYLATION IN HUMAN RED CELLS

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In inside-out red cell membrane vesicles ATP-dependent calcium transport is activated by the divalent metal ions Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+} and Fe^{2+}. This activation is based on the formation of Me^{2+}-ATP complexes which can serve as energy-donor substrates for the calcium pump, and probably, satisfy the requirement for free Me^{2+} in this transport process. Higher Me^{2+} concentrations inhibit calcium transport with various efficiencies. Mn^{2+} directly competes with Ca^{2+} at the transport site, while other divalent metal ions investigated have no such effect. The formation of the hydroxylamine-sensitive phosphorylated intermediate (EP) of the red cell membrane calcium pump from [γ-^{32}P]ATP is induced by Ca^{2+} while rapid dephosphorylation requires the presence of Mg^{2+}. At higher concentrations Mn^{2+} and Ni^{2+} inhibit predominantly the formation of EP, while Co^{2+} and Fe^{2+} block dephosphorylation. The possible sites and nature of the divalent metal interactions with the red cell calcium pump are discussed. Hydroxylamine-insensitive membrane phosphorylation in inside-out vesicles from [γ-^{32}P]ATP is significantly stimulated by Mn^{2+} and Co^{2+}, as compared to that produced by Mg^{2+}, Fe^{2+} and Ni^{2+}. Part of this labelling is found in phospholipids, especially in phosphatidylinositol. The results presented for the metal dependency of protein and lipid phosphorylation in red cell membranes may help in the characterization of ATP consumptions directly related to the calcium pump and those involved in various regulatory processes.

Introduction

Active calcium transport is a basic function of animal cell membranes [1–3], and human red cells may serve as model systems for its investigation. The preparation of inside-out red cell membrane vesicles allows the simultaneous study of calcium transport, (Ca^{2+} + Mg^{2+})-ATPase activity and membrane phosphorylation reactions [3,4]. As we recently reported [5], several divalent metal ions activate calcium pumping in inside-out vesicles, with various efficiencies; that is, the maximum calcium transport rates produced and the metal concentrations required for half-maximum activation ($K_{Me^{2+}}$) are considerably different. Calmodulin, the cytoplasmic modulator of the red cell calcium pump, exerts its stimulatory effect practically independent of the divalent metals used for activating calcium transport [5]. In the first part of the present paper we further characterize the nature of Me^{2+}-pump interactions, while in the second part we report experiments on the metal-induced changes in inside-out vesicle membrane phosphorylation, which are apparently not (directly) related to the function of the calcium pump.

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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Materials and Methods

All the chemicals used were of analytical grade. [γ-^{32}P]ATP with a specific activity of 600 GBq/mmol was purchased from Amersham International, Amersham, U.K.

Inside-out vesicles were prepared as described previously [4]. After preparation they were washed with 10 vol. of 10 mM Tris-HCl + 2 mM EDTA and twice with 10 mM Tris-HCl (pH 7.0 at 0°C, 10-min centrifugations at $20000 \times g$). The final pellet was resuspended to obtain a 4–5 mg protein/ml solution in 0.16 M KCl buffered with 5 mM Tris-HCl to pH 7.4. The percentage of sealed inside-out vesicles was between 72 and 78 in all preparations.

Calcium influx into vesicles was measured by the Sartorius membrane-filtration method as described in Refs. 4 and 6. Each data point represents triplicate experiments by measuring inside-out vesicle calcium transport in 3-min incubation periods at 37°C.

Phosphorylation of inside-out vesicles was carried out in reaction media containing 130 mM KCl, 17 mM Hepes, 20 μM $CaCl_2$; $MgCl_2$, cobalt acetate, $NiSO_4$ or $FeCl_2$ as indicated, and 0.25–0.5 mg inside-out vesicle protein in a final volume of 0.5 ml (pH 7.0 at 0°C). The reaction was initiated by the addition of [γ-^{32}P]ATP to obtain a final concentration of 0.5 μM. The tubes were incubated for 20 s–5 min at 0°C and the reaction was terminated by the addition of 5 ml of ice-cold 6% trichloroacetic acid containing 1 mM ATP and 10 mM P_i.

For the dephosphorylation experiments, phosphorylation was carried out for 20 s at 0°C and the membrane was chased by the rapid addition of 0.5 ml of a solution containing 20 μM ATP. After 7 s the reaction was terminated with the stopping solution. The pellet was collected by centrifugation, and washed three times with 10 ml of the trichloroacetic acid/ATP/P_i solution. The washed precipitate was dissolved in 1% sodium dodecyl sulfate (SDS), and the amount of ^{32}P incorporated was measured in an Intertechnique SL-30 Liquid scintillation spectrometer.

Hydroxylamine treatment of the phosphorylated proteins was carried out according to Rega et al. [7].

SDS-polyacrylamide gel electrophoresis was performed according to Fairbanks et al. [8] with a current of 8–10 mA/tube. Acrylamide concentration was 5.6%. The gels were cut into 2.5-mm slices for measuring radioactivity.

Lipids were extracted from the trichloroacetic acid-washed pellets by 10 ml of chloroform/methanol/HCl (100:100:0.6, v/v) as described by Schneider et al. [9]. The lipid extracts were partitioned by adding 3 ml of 1 M HCl and the two phases were separated by low speed centrifugation. The upper phase and the interfacial protein fraction were discarded. The chloroform phase was washed with a solution of chloroform/methanol/0.2 M HCl (3:47:50, v/v). The washed chloroform extract was evaporated to dryness.

The extracted phospholipids were separated by one-dimensional thin-layer chromatography (TLC) on glass plates coated with 0.5 mm of Merck Silica H according to Weiss and Putney [10]. Plates were sprayed with a solution containing 10% $HClO_4$, 50% ethanol and 5% phosphomolybdenic acid. Lipid spots were visualized by heating for 10 min at 130°C, and the amounts of stained phospholipids were quantified by scanning in a Telechrom 007 Videodensitometer. Phosphatidylinositol and phosphatidic acid, chromatographed on the same plates, were used as standards.

For measurements of ^{32}P incorporation, the gel were scraped from the TLC plates, and radioactivity was determined in a liquid-scintillation spectrometer.

For the calculations of different MeATP concentrations the following stability constants were used: $10^{4.0}$ for MgATP, $10^{4.9}$ for MnATP and $10^{3.9}$ for CaATP [11].

Protein concentration was determined by the method of Lowry et al. [12].

Results

A. Calcium transport experiments

The data presented in Fig. 1 show the effects of various divalent cations on the rate of active calcium uptake into inside-out vesicles. In order to minimize the chelation of metal ions, in contrast to the previously applied imidazole-HCl buffer [5] here we used a Hepes-K buffer. The dependence of calcium pumping on Mg^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+},

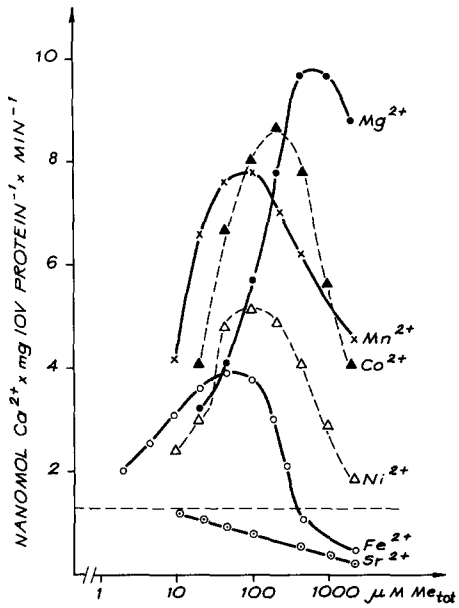

Fig. 1. Effects of divalent metal ions on the rate of active calcium uptake by inside-out vesicles. The incubation media contained 130 mM KCl, 17 mM Hepes-K (pH 7.0), 20 μM $CaCl_2$ (including ^{45}Ca tracer), 20 μM ATP, and the divalent metal ions in the total concentrations indicated. When using Fe^{2+}, the media contained 1 mM ascorbic acid to keep this ion in its reduced form. Inside-out vesicle (IOV) concentration 20 μg/ml medium, temperature 37°C.

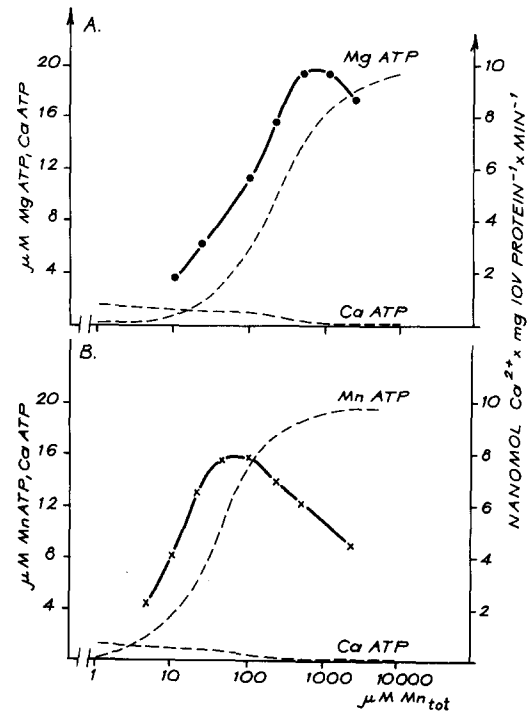

Fig. 2. Correlation of active calcium transport rate with the concentration of Mg-ATP (A) or Mn-ATP (B) complexes. For the incubation conditions see the legend of Fig. 1. The dashed lines represent computed concentrations of MgATP, MnATP and CaATP, respectively. IOV, inside-out vesicle.

or Ni^{2+} concentrations is biphasic (lower metal concentrations activate, while higher concentrations inhibit calcium transport). As demonstrated in Fig. 1, maximum calcium transport rates, as well as half-maximum activating and inhibiting concentrations, are significantly different when using different divalent metal ions. It is important to note that Sr^{2+}, Ba^{2+}, Fe^{3+} and La^{3+} do not have any activating effect on inside-out vesicle calcium transport.

Fig. 2 shows the calculated concentrations of MgATP, MnATP and CaATP, respectively, and the measured rates of calcium transport into inside-out vesicles, at various total Mg^{2+} and Mn^{2+} concentrations. The increase observed in the calcium transport rate is essentially parallel to the increase in MgATP or MnATP, while CaATP almost completely disappears at the maximum activation of calcium uptake. A similar figure can be obtained for CoATP, while increasing concentrations of Ni^{2+} and Fe^{2+} inhibit calcium transport so strongly that such a comparison cannot be properly made (data not shown).

The above data indicate that stimulation of calcium uptake by Mg^{2+}, Mn^{2+}, or Co^{2+} is basically produced by the increase in specific Me^{2+}-ATP complex concentrations which serve as energy-donor substrates for the calcium pump. Ni-ATP and Fe-ATP complexes apparently are also accepted as substrates of this transport system.

In the following we analyze the type of interaction of various divalent metal ions with the calcium pump enzyme. Fig. 3 shows the calcium concentration dependence of active calcium transport in the presence of Mg^{2+}, Mn^{2+}, Co^{2+} and Fe^{2+}. When Mg^{2+}, Co^{2+} or Fe^{2+} were used as activators of calcium uptake the value of K_{Ca} was practically the same (30–33 μM) and only the V_{max} of the calcium transport varied. In the case of Mn^{2+} we

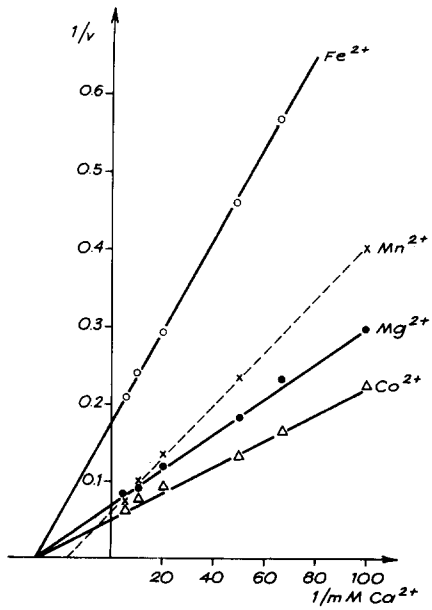

Fig. 3. Calcium concentration dependence of active calcium uptake in inside-out vesicles. Double reciprocal plot. Mean values from three separate experiments. The change in K_{Ca} caused by Mn^{2+} was found to be significant. Calcium transport was measured as described in the legend for Fig. 1. The Me^{2+} concentrations were as follows: 500 μM Mg^{2+} (●———●); 200 μM Fe^{2+} (○———○); 200 μM Mn^{2+} (×------×); 200 μM Co^{2+} (△———△). V is expressed as nmol Ca^{2+}·(mg IOV protein)$^{-1}$·min^{-1}.

found a variable value for K_{Ca}, and at 0.2 mM Mn^{2+} K_{Ca} was about 55–60 μM. As these experiments were carried out in practically calmodulin-free (EDTA-washed) inside-out vesicles, the double-reciprocal plots were linear (in contrast to a biphasic characteristic observed previously in EDTA-untreated inside-out vesicles, see ref. 6).

B. Membrane phosphorylation experiments

Fig. 4 demonstrates the effect of metal ions on the hydroxylamine-sensitive and insensitive phosphorylations by [γ-^{32}P]ATP in inside-out vesicle membranes. As we previously showed [13] and present here in Fig. 5A, under the conditions applied the NH_2OH-sensitive, calcium-induced phosphorylation involves the labelling of one single polypeptide with a molecular weight of 140000–150000, which is the phosphorylated intermediate of the red cell membrane calcium pump [7,14–16].

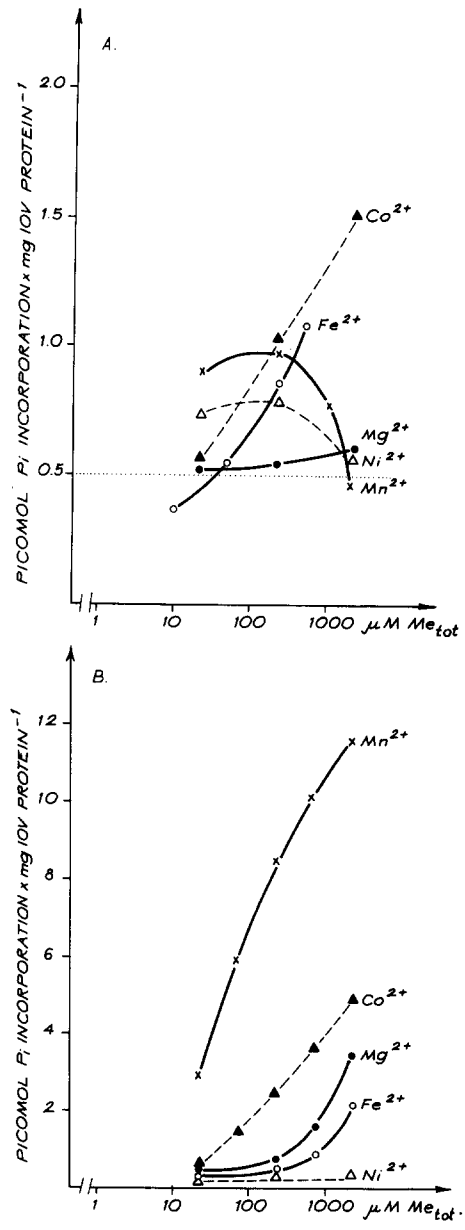

Fig. 4. Effects of divalent metal ions on the membrane phosphorylation in inside-out vesicles. Phosphorylation was carried out at 0°C for 20 s, in media containing 130 mM KCl, 17 mM Hepes-K (pH 7.0), 20 μM $CaCl_2$, 0.5 μM [γ-^{32}P]ATP and the divalent metals as indicated. When investigating the effect of Fe^{2+}, the media contained 1 mM ascorbic acid, which had no effect on phosphorylation in the presence of 20 μM Ca^{2+}. Inside-out vesicle (IOV) concentration was 0.5 mg/ml medium. (A) Hydroxylamine-sensitive EP-formation of the calcium pump. The dotted line represents ^{32}P incorporation in the presence of 20 μM Ca^{2+} in the absence of other metals. (B) Hydroxylamine-insensitive membrane phosphorylation. For hydroxylamine treatment see Materials and Methods.

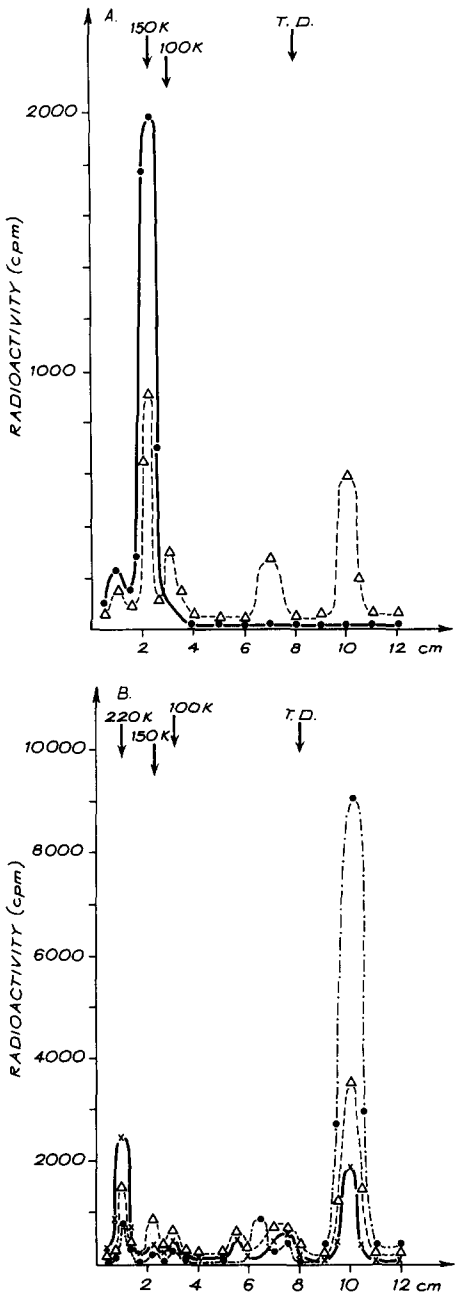

Fig. 5. SDS-polyacrylamide gel electrophoretogram of inside-out vesicle membranes phosphorylated by [γ-^{32}P]ATP. For the conditions of phosphorylation see the legend to Fig. 4. (A) Inside-out vesicles phosphorylated for 20 s in the presence of 20 μM La^{3+} (●———●), and 20 μM Ca^{2+} +2 mM Co^{2+} (△------△). (B) Inside-out vesicles were phosphorylated for 5 min in the presence of 20 μM Ca^{2+} +2 mM Mn^{2+} (●-·-●); 20 μM Ca^{2+} +2 mM Co^{2+} (△------△); and 20 μM Ca^{2+} +2 mM Mg^{2+} (×———×). T.D., tracking dye.

(A minor ^{32}P incorporation is usually observed in a high molecular weight fraction, which is probably a dimer of the pump enzyme, see Ref. 16.) The data in Fig. 4A represent steady-state values for EP, obtained at 0°C in 20-s incubation periods. This steady-state value is significantly enhanced by lanthanum (see Fig. 5A), which inhibits calcium pumping by blocking the dephosphorylation reaction [15,17]. The consistent increase in EP level with increasing concentrations of Co^{2+} and Fe^{2+} suggests that these metals also inhibit the hydrolysis of EP. The effect of Mn^{2+} is biphasic: lower Mn^{2+} concentrations (10–100 μM) increase, while higher Mn^{2+} concentrations decrease the level of EP. The inhibition of EP formation by high Mn^{2+} concentrations is most probably caused by a direct competition between this metal ion and calcium at its specific binding site(s) (see Fig. 3).

In order to investigate this problem further we tested the effects of metal ions on the dephosphorylation of EP in inside-out vesicles (Table I). In these experiments we initiated the decay of ^{32}P-labelled EP by diluting the medium with unlabelled ATP. If 500 μM EDTA were added to the

TABLE I

EFFECTS OF DIVALENT METAL IONS ON THE DEPHOSPHORYLATION OF THE CALCIUM-DEPENDENT PHOSPHOENZYME IN INSIDE-OUT VESICLES

The phosphorylation reaction was carried out in the presence of 20 μM Ca as in Fig. 4, without any other metals added to the media. After 20 s of incubation at 0°C, equal volumes of different chase solutions containing 20 μM ATP were added. Dephosphorylation was stopped after 7 s. Results are means ± S.D. from three separate experiments.

Additions in the chase solutions	% of EP remaining
500 μM EDTA	90±3
None	42±4
500 μM Mg^{2+}	40±3
20 μM Mn^{2+}	52±4
500 μM Mn^{2+}	50±5
20 μM Co^{2+}	49±3
500 μM Co^{2+}	59±4
2 mM Co^{2+}	75±4
50 μM Fe^{2+}	44±3
500 μM Fe^{2+}	65±4
50 μM Ni^{2+}	44±3
2 mM Ni^{2+}	50±4

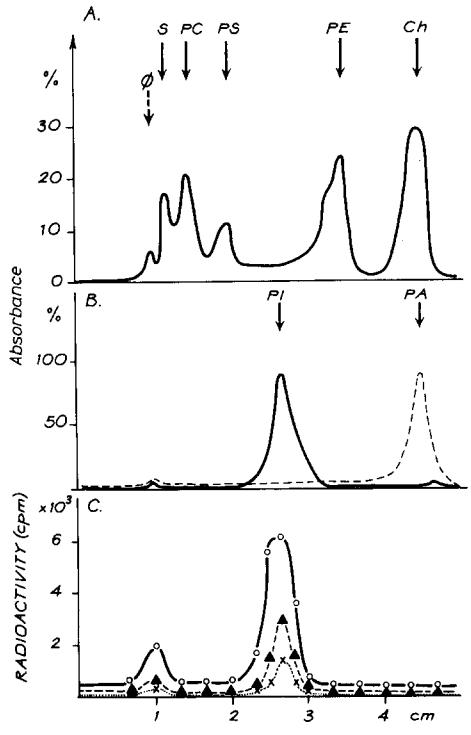

Fig. 6. Separation of the labelled phospholipids in thin-layer chromatography. (A) Thin-layer chromatogram of phospholipids extracted from inside-out vesicles. Extraction and chromatography of phospholipids were carried out as described in Materials and Methods. Key: Ø, origin front; S, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; Ch, cholesterol. (B) Thin-layer chromatogram of phosphatidylinositol (PI) and phosphatidic acid (PA) used as standards. (C) ^{32}P radioactivity on TLC plates. Inside-out vesicles were phosphorylated as described in the legends for Fig. 4 and Fig. 5B. The phosphorylation media contained 20 μM Ca^{2+} +2 mM Mn^{2+} (○——○); 20 μM Ca^{2+} +2 mM Co^{2+} (▲--▲); and 20 μM Ca^{2+} +2 mM Mg^{2+} (×·····×). For the conditions of measurements see Materials and Methods.

chase solution, there was no significant decrease in the EP level (the time for dephosphorylation measurements was 7 s). In the absence of EDTA the rate of decay was too fast to see any acceleration produced by the added metals, but an inhibition of the dephosphorylation reaction could be observed by higher concentrations of Co^{2+} and Fe^{2+}. These experiments, in accordance with the data in the literature [18], suggest that membrane-bound Mg^{2+} suffices for EP decomposition. Therefore, in such a system we could not demonstrate whether Mn^{2+}, Co^{2+}, Fe^{2+} and Ni^{2+} can replace Mg^{2+} in this role.

Part B of Fig. 4 shows the metal-concentration dependence of hydroxylamine-insensitive membrane phosphorylations in inside-out vesicles. Mn^{2+}, Co^{2+} and Mg^{2+} strongly enhance this membrane phosphorylation, whereas Ca^{2+} and Ni^{2+} have no such effect. As demonstrated in Fig. 5B, the actual labelling of membrane constituents, when examined by SDS-polyacrylamide gel electrophoresis, is significantly different when using different metal ions. In the presence of Mg^{2+} the most pronounced labelling occurs in the 220000–240000 mol. weight 'spectrin' and in the 90000–100000 mol. weight 'band 3', while Mn^{2+} induces predominantly the labelling of a low molecular weight fraction. As in this latter fraction the gels

TABLE II

^{32}P RADIOACTIVITY IN INSIDE-OUT VESICLE PROTEINS AND PHOSPHOLIPIDS

Phosphorylation was carried out for 5 min. For the incubation conditions see the legend of Fig. 4 and 5B. Results are means ±S.D. from three different experiments. Figures in the three columns using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) do not add up to 100%. The remaining radioactivity was found in various, non-identified fractions.

Conditions for phosphorylation	% radioactivity in SDS-PAGE			% Radioactivity extracted in lipids
	Spectrin	Band 3	Low mol. weight peak	
2 mM Mn^{2+} +20 μM Ca^{2+}	8±2	5±1	69±4	62±4
2 mM Co^{2+} +20 μM Ca^{2+}	19±3	11±2	36±3	36±3
2 mM Mg^{2+} +20 μM Ca^{2+}	34±3	10±2	26±2	22±2

showed no protein-staining, we suspected the labelling of membrane phospholipids. Phospholipid-extraction experiments showed that this was indeed the case: the ^{32}P labelling in the low molecular weight fractions could be quantitatively extracted by acidic chloroform-methanol solutions (Table II)(no labelled lipids could be extracted at neutral pH).

Fig. 6 shows the qualitative analysis of the labelled phospholipids in thin-layer chromatography: the only lipid fraction containing ^{32}P radioactivity was phosphatidylinositol. (A variable 20–30% of radioactivity was found at the starting point, probably as a consequence of overloading of the plates with unlabelled phospholipids and a partial dephosphorylation during extraction and chromatography.)

Discussion

ATP-dependent active calcium transport requires the presence of calcium and magnesium at the internal membrane surface; the molecular basis of this transport is a $(Ca^{2+}+Mg^{2+})$-activated ATPase. As we recently reported in a short communication [5], magnesium can be replaced in this role by several divalent cations, such as Mn^{2+}, Co^{2+}, Fe^{2+} and Ni^{2+}. These metals (similarly to magnesium) are not transported by the calcium pump but their interactions with the pump protein and with other membrane constituents may substantially increase our understanding of the molecular mechanism of active calcium transport.

In the past few years the energy-donor substrate specificity of the red cell calcium pump has been widely discussed in the literature. Opinions diverged whether the true, physiological substrate is MgATP [19,20], free ATP [21,6] or CaATP [22,23]. In our recent papers [5,24] we have shown experiments indicating that the physiological substrate of the in situ red cell calcium pump is MgATP. The data presented here further substantiate the conclusion that the best accepted substrate of the pump is a specific Me^{2+}-ATP complex. At fixed total Ca^{2+} and ATP concentrations the activation of the calcium transport by Mg^{2+}, Mn^{2+} and Co^{2+} is essentially parallel with an increase in the given Me^{2+}-ATP concentration, which makes the suggestion unlikely that the favourable substrate of the pump would be either Ca-ATP or free ATP. In sarcoplasmic reticulum calcium transport the physiological substrate is most probably also MgATP [25] and Mn^{2+} or Co^{2+} can certainly substitute for Mg^{2+} in this complex formation [26,27].

As shown in Fig. 1, divalent metals in higher concentrations inhibit active calcium transport with various effectivity. It has already been indicated in the literature that metal ions may interact with the calcium transport site of the pump and inhibit ATPase or transport activity [2,3,6,15]. In order to study this interaction we examined the calcium concentration dependence of the pump at various concentrations (including inhibitory levels) of the applied metals. Mn^{2+} significantly reduced the apparent calcium affinity of the enzyme (K_{Ca} increased from about 30 to about 60 μM in the presence of 0.2 mM Mn^{2+}), while we did not observe such a change in the presence of Mg^{2+}, Co^{2+}, Fe^{2+} or Ni^{2+}. Thus an $Mn^{2+}-Ca^{2+}$ competition could be observed at the calcium-specific binding site(s), while other metals probably interact with other sites of the pump enzyme. The most likely candidate for such an interaction is the site where free Mg^{2+} is required to activate calcium transport and ATPase activity. It has been shown that calcium-induced formation of a phosphorylated intermediate (EP) of the red cell calcium pump from ATP does not require the presence of Mg^{2+}, while EP decomposition is significantly accelerated by magnesium [7,15,17,18,28]. Since EP dephosphorylation is probably one of the rate-limiting steps of the pump cycle, free magnesium is required for its rapid completion (see Refs. 2 and 3).

When examining the effects of metal ions on EP formation from [γ-^{32}P]ATP, we found an increased steady-state EP level at increasing Co^{2+} and Fe^{2+} concentrations (a maximum labelling of EP could be produced in the presence of La^{3+}, a known inhibitor of EP hydrolysis [15,17]). Combining these data with those from the measurment of EP-dephosphorylation (Table I), we suggest that Co^{2+} and Fe^{2+} directly inhibit this latter reaction, possibly by competing with Mg^{2+} at this site. The biphasic action of Mn^{2+} on the EP level is probably due to a combination of the inhibition of EP hydrolysis (above 50–100 μM) with a competition

with calcium in inducing EP formation (effective above 200 μM). Inhibition of calcium transport by Ni^{2+} (above 200 μM) and by extremely high concentrations of Mg^{2+} (10 mM) is probably due to a non-specific interaction between these metals and the pump protein. Such an inhibitory effect of Mg^{2+} is much more pronounced in the 'naked', purified calcium pump protein [22,23].

The question of whether free Mg^{2+} in stimulating dephosphorylation can be replaced by another divalent metal could not be resolved in the present study. Since enzyme-bound Mg^{2+} is sufficient for this role, the technique applied does not allow a definite answer. A partial stimulation of red cell membrane ($Ca^{2+} + Mg^{2+}$)-ATPase by various metals, as shown by Pfleger and Wolf [29], was most probably caused by an interaction of these ions with the magnesium-binding site of the pump.

In the course of the membrane phosphorylation studies we observed a significant increase in the hydroxylamine-insensitive labelling, that is the production of phosphate-ester bonds, by [γ-^{32}P]ATP at increasing Me^{2+} concentrations. Mn^{2+} and Co^{2+} proved especially effective in producing such a ^{32}P incorporation, while Ca^{2+}, Ni^{2+} or La^{3+} did not induce this phenomenon. Based on gel-electrophoresis and lipid-extraction studies we could identify the phosphate labelling in various proteins, predominantly in Spectrin 2, and in the phospholipid phosphatidylinositol. Various metals induced various patterns of labelling: spectrin phosphorylation was the most pronounced in the presence of high concentrations (5–10 mM) of Mg^{2+}, while phosphatidylinositol was most effectively labelled in the presence of Mn^{2+}. Based on these results we can certainly conclude that specific protein and lipid kinases are present in our membrane preparation, and various metals either stimulate kinase activity or inhibit phosphatase activity in a fairly selective manner. The regulatory role of spectrin phosphorylation–dephosphorylation in red cell shape changes [30,31] or in mediating adrenergic responses [32], as well as the possible regulatory role of phosphorylated forms of phosphatidylinositol in cell shape or some membrane-transport processes [31,33–37] have been suggested in the literature. A spectrin-dependent, 'high calcium affinity' ATPase in red cell membranes has recently been observed [38], and the inhibition of spectrin kinase activity by calcium also reported [39]. The observed specific activations by specific metal ions may open new perspectives in studying these processes. The kinetic separation of phosphorylation–dephosphorylation reactions is certainly required for any further investigation of the role of calcium and other metal ions in these phenomena. The combined protein and/or lipid kinase + phosphatase reactions may add up to produce a pump-independent ($Ca^{2+} + Mg^{2+}$)-ATPase activity, as found in red cell ghosts [40] or in intact red cells [41], and the different effects of divalent cations on these processes may help in distinguishing such reactions from the calcium pump activity.

An especially intriguing possibility is the direct involvement of phosphatidylinositol phosphorylation in the regulation of membrane calcium transport. Studies for exploring some of the above-mentioned possibilities are currently under way in our laboratory.

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