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IDENTIFICATION OF Ca^{2+}-PUMP-RELATED PHOSPHOPROTEIN IN PLASMA MEMBRANE VESICLES OF EHRLICH ASCITES CARCINOMA CELLS

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Plasma membrane vesicles of Ehrlich ascites carcinoma cells have been isolated to a high degree of purity. In the presence of Mg^{2+}, the plasma membrane preparation exhibits a Ca^{2+}-dependent ATPase activity of 2 μmol P_i per h per mg protein. It is suggested that this $(Ca^{2+} + Mg^{2+})$-ATPase activity is related to the measured Ca^{2+} transport which was characterized by K_m values for ATP and Ca^{2+} of 44 ± 9 μM and 0.25 ± 0.10 μM, respectively. Phosphorylation of plasma membranes with [γ-^{32}P]ATP and analysis of the radioactive species by polyacrylamide gel electrophoresis revealed a Ca^{2+}-dependent hydroxylamine-sensitive phosphoprotein with a molecular mass of 135 kDa. Molecular mass and other data differentiate this phosphoprotein from the catalytic subunit of $(Na^+ + K^+)$-ATPase and from the catalytic subunit of $(Ca^{2+} + Mg^{2+})$-ATPase of endoplasmic reticulum. It is suggested that the 135 kDa phosphoprotein represents the phosphorylated catalytic subunit of the $(Ca^{2+} + Mg^{2+})$-ATPase of the plasma membrane of Ehrlich ascites carcinoma cells. This finding is discussed in relation to previous attempts to identify a Ca^{2+}-pump in plasma membranes isolated from nucleated cells.

Introduction

Since the discovery and purification of the $(Ca^{2+} + Mg^{2+})$-ATPase of human erythrocyte membranes [1–3], numerous attempts have been undertaken to identify Ca^{2+}-transporting enzymes in plasma membranes of nucleated cells. The presence of Ca^{2+}-stimulated ATPase and Ca^{2+} transport activity has been reported for plasma membrane preparations of several cell types [4–7]. To relate the $(Ca^{2+} + Mg^{2+})$-ATPase and Ca^{2+} transport activities to a plasma membrane bound Ca^{2+} pump it is necessary to differentiate the presumed enzyme from the well characterized Ca^{2+}-pump of endoplasmic reticulum. The latter was shown to consist of a catalytic subunit with a molecular mass of 100 kDa [8,9]. Therefore, one method of differentiation would be the identification of a Ca^{2+}-dependent phosphoprotein with a molecular mass different from 100 kDa.

Hitherto, investigations have been focused on the identification of the $(Ca^{2+} + Mg^{2+})$-ATPase of myocardial plasma membranes. Because of the large amount of reticulum in these cells, the exclusion of sarcoplasmic contamination in preparations of myocardial plasma membranes is extremely difficult. As described in a preceding paper [10] and in accordance with others [11], we did not

Abbreviations Cl-CCP, carbonylcyanide-*m*-chlorophenylhydrazone; EGTA, ethyleneglycolbis(β-aminoethylether)*N*,*N*′-tetraacetic acid; PMSF, phenylmethylsulphonyl fluoride; SDS, sodium dodecylsulphate.

succeed in finding a Ca^{2+}-dependent phosphoprotein in sarcolemma membranes unequivocally different from that described for the catalytic subunit of $(Ca^{2+} + Mg^{2+})$-ATPase of sarcoplasmic reticulum. Using calmodulin affinity chromatography for the first time, Caroni and Carafoli [12] succeeded in demonstrating the phosphoprotein of heart sarcolemma $(Ca^{2+} + Mg^{2+})$-ATPase of 150 kDa.

In this paper we describe $(Ca^{2+} + Mg^{2+})$-ATPase activities, Ca^{2+} transport properties and Ca^{2+}-dependent phosphorylation by use of highly purified plasma membrane vesicles from Ehrlich ascites carcinoma cells. We could demonstrate a Ca^{2+}-dependent phosphoprotein (molecular mass 135 kDa) in native plasma membranes from Ehrlich ascites carcinoma cells which is unambiguously different from the catalytic subunits of the $(Na^+ + K^+)$-ATPase of the plasma membrane and of the reticular $(Ca^{2+} + Mg^{2+})$-ATPase. This finding clearly points to the existence of a plasma membrane bound $(Ca^{2+} + Mg^{2+})$-ATPase in Ehrlich ascites carcinoma cells.

Materials and Methods

Chemicals

$^{45}CaCl$ (1.33 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from Amersham International (U.K.), substrates and enzymes from Reanal (Hungary), reagents for polyacrylamide gel electrophoresis were from Serva (F.R.G.), PMSF from Sigma (U.S.A.) and Cl-CCP from Calbiochem (U.S.A.) Salts were suprapur from Merck (F.R.G.) and all other reagents were analytical grade. Calmodulin was a kind gift from Dr. J. Kyte.

The sodium salt of ATP was converted into the imidazole form using the ion-exchanger CM-Sephadex C-25.

Isolation of plasma membranes

Ehrlich ascites carcinoma cells (hyperdiploid strain) were grown in female AB mice and harvested 13–15 days after inoculation. The cell suspension was diluted about 10-fold with a solution consisting of 20 mM imidazole/0.15 M NaCl (pH 7.4) (buffered saline) immediately after aspiration. Cells from up to eight mice were collected and washed 2–3-times by centrifugation at $300 \times g$ for 5 min at room temperature with buffered saline. $(0.5–1.0) \cdot 10^9$ washed cells were obtained per mouse.

The method developed for the isolation of plasma membranes follows principles outlined by Forte et al. [13], Im et al. [14] and Kilberg and Christensen [15].

All steps were performed at 0–4°C. All solutions except the sucrose/Ficoll mixtures were made $1 \cdot 10^{-4}$ M in PMSF immediately before use.

Sedimented cells from 6–8 mice were suspended in 100 ml 15 mM imidazole/15 mM NaCl/1 mM $MgCl_2$/0.3 mM dihiothreitol (pH 7.4) (homogenization buffer) and the cells were swollen at 0°C for 20 min. Cell breakage was performed with 5–15 strokes in a tight-fitting Dounce homogenizer monitored by phase-contrast microscopy until only a few intact cells were left and yet no damage of nuclei was observable. The homogenate was diluted to about 300 ml with the homogenization buffer and centrifuged at $200 \times g$ for 5 min. The pellet was resuspended in fresh homogenization buffer and spun down at $200 \times g$ for 5 min, then the combined supernatants were again centrifuged under the same conditions. The final supernatant was carefully decanted and was centrifuged at $3000 \times g$ for 15 min. The resulting pellet was suspended in a solution containing 20 mM imidazole/1 mM EDTA (pH 8.0) and passed twice through a 0.7×30 mm (No. 12) syringe needle. The suspension was filled up to 30 ml and 5-ml aliquots were layered on top of discontinuous gradients formed by underlayering 29 ml 0.8 M sucrose/3% (w/v) Ficoll with 3 ml 0.8 M sucrose/10.4% Ficoll. The gradients were centrifuged at $100\,000 \times g$ for 1 h in an SW27 rotor. The plasma membrane fraction appeared as a narrow double band at the interphase between the imidazole buffer on top and the 3% Ficoll/sucrose solution and was harvested with a Pasteur pipette. The membranes were diluted with medium consisting of 0.3 M sucrose/20 mM imidazole/0.3 mM dithiothreitol (pH 7.3), pelleted at $100\,000 \times g$ for 20 min and washed once under the same conditions with the dilution nuffer. If not processed further, the membranes were stored in the dilution medium at a protein concentration of 1–4 mg per ml under liquid nitrogen.

Determination of enzymatic activities

$(Na^+ + K^+)$-ATPase was determined at 37°C as described by Jørgensen [16]. Incubation of 25–50 μg membrane protein was performed for 15 min in 0.5 ml of a reaction medium composed of 3 mM $MgCl_2$/130 mM NaCl/20 mM KCl (complete medium) in 30 mM histidine-HCl buffer (pH 7.5). The reaction was started by addition of ATP to a final concentration of 3 mM and terminated by addition of trichloroacetic acid to a final concentration of 5%. Inorganic phosphate was estimated by the method of Lohmann and Langen [17]. The value for $(Na^+ + K^+)$-ATPase activity was obtained as the difference between ATPase activity in the absence or presence of 1 mM oubain, or alternatively as the difference between activities in the presence or absence of Na^+. For disruption of the vesicles and measuring the maximal $(Na^+ + K^+)$-ATPase activity, the incubation mixture was either quickly frozen and thawed or preincubated for 15 min in the presence of 1 mM deoxycholate (resulting in deactivation of the total ATPase by 30–40%).

$(Ca^+ + Mg^{2+})$-ATPase activity was determined at 30°C by means of the coupled test system described by Schwartz [18]. For measurement of $(Ca^{2+} + Mg^{2+})$-ATPase activity under ionic conditions of transport (see below), the reaction medium (1 ml) comprised 0.3 M sucrose, 100 mM KCl, 5 mM $MgCl_2$, 0.3 mM dithiothreitol and about 20 μg of plasma membrane protein in 20 mM imidazole-HCl buffer (pH 7.3). Measurement of $(Ca^{2+} + Mg^{2+})$-ATPase after disruption of vesicular structure was performed by freezing and thawing of plasma membranes in the described reaction medium in the absence of sucrose. Before starting the reaction with 3mM ATP (final concentration) the medium was completed by addition of 2 IU pyruvate kinase and 2 IU lactate dehydrogenase.

The oxidation of NADH was monitored at 340 nm. The difference between ATPase activity measured in the presence of 20 μM Ca^{2+} and, alternatively, in presence of 500 μM EGTA, yielded the Ca^{2+}-dependent ATPase activity.

Succinate dehydrogenase and β-glucuronidase were estimated according to the methods of Earl and Korner [19] or Fishman [20], respectively. Glucose-6-phosphatase was estimated as described in Ref. 21.

Ca^{2+} transport measurements

Accumulation of calcium into plasma membrane vesicles was determined in an incubation medium (final volume 2.1 ml) containing 100 mM KCl, 5 mM $MgCl_2$, 0.3 mM dithiothreitol, 5 mM phospho*enol*pyruvate, 2IU pyruvate kinase, 2 mM ATP, 5 μM Cl-CCP, 36.8 μM $^{45}CaCl$ (approx. 200 dpm per pmol ^{45}Ca), 0.3 M sucrose and 20 mM imidazole-HCl buffer (pH 7.3). The desired concentration of ionized Ca was fixed by addition of EGTA ($K_{d(CaEGTA)} = 0.15$ μM [22]).

The incubation medium was preincubated at 30°C for 2 min and the reaction was started by addition of 210 μg freshly prepared plasma membranes. After various time intervals, 0.4 ml aliquots were withdrawn, immediately filtered through Millipore filters, type GS (0.45 μm pore size) and washed twice with ice-cold 1 mM EGTA/100 mM KCl/5 mM $MgCl_2$/0.3 M sucrose/20 mM imidazole-HCl buffer (pH 7.3). The radioactivity was measured in Bray scintillator using a Philips liquid scintillation counter.

All measurements were carried out in duplicate or triplicate. Each experiment has been repeated with at least three different vesicle preparations.

Phosphorylation of plasma membrane proteins with [γ-^{32}P]ATP

Phosphorylation of plasma membranes was carried out at 0°C in 1.0 ml of a medium containing 36.8 μM $CaCl_2$, 16.9 μM EGTA, 5μM Cl-CCP, 20 mM imidazole-HCl buffer (pH 7.3) and 500–800 μg membrane protein. The concentration of uncomplexed Ca^{2+} was calculated to be 20 μM. The concentrations of $MgCl_2$ and other constituents, if added, are given in the legends to the figures.

The phosphorylation reaction was started by addition of [γ-^{32}P]ATP to a final concentration of 1.3–2 μM (approx. 30000 dpm/pmol). After 20 s the reaction was stopped by the addition of 4 ml ice-cold 6% trichloroacetic acid/50 mM H_3PO_4/0.5 mM ATP. Protein was sedimented by centrifugation at 6000 × *g* for 10 min and washed three times with 5 ml 6% trichloroacetic acid/50 mM H_3PO_4. The pellet was resuspended in 0.2 ml of a solubilization mixture consisting of 10% glycerol/5% mercaptoethanol/1 mM EDTA/2% (w/v) SDS and was saturated with PMSF. After incubation at 30°C for 3 min the solubilized mem-

brane proteins were immediately submitted to electrophoretic separation.

To prove the effect of hydroxylamine, the phosphorylated and washed membranes were suspended in 4 ml 0.08 M sodium acetate (pH 5.6) with 0.8 M hydroxylamine or, as a control, with 0.8 M NaCl. After incubation for 10 min at 20°C the protein was precipitated with 1ml 50% trichloroacetic acid and further processed for electrophoresis as described above.

Gel electrophoresis and autoradiography

SDS-polyacrylamide gel electrophoresis in phosphate buffer at pH 2.4 on cylindrical or slab gels (5.6% acrylamide) was performed according to Avruch and Fairbanks [23].

Gels were stained with Coomassie brilliant blue as described by Fairbanks et al. [24] or analyzed for distribution of radioactivity. In the case of cylindric gels, these were cut into 1 mm disks. Cerenkov radiation of each disk was counted in 5 ml 0.5 M NaOH. For autoradiographic localization of ^{32}P-labeled protein bands, slab gels were fixed for 30 min in 50% methanol/10% acetic acid, washed with 98% methanol for 30 min and then with 3% glycerol for 3–4 min. After drying on paper the slabs were exposed to ORWO XR-1 X-ray film.

Molecular masses of proteins were calculated calibrating the gels with the following standard proteins: catalytic subunit of $(Na^+ + K^+)$-ATPase from pig kidney, highly purified exactly as described by Jørgensen [25] (100 kDa): bovine serum albumin (monomer 68 kDa, dimer 136 kDa): aldolase (40 kDa) and cytochrome *c* (12.5 kDa).

Estimation of protein

Protein was determined according to the method of Lowry et al. [26] with bovine serum albumin as standard.

Results

Characterization of plasma membranes

The electron microscopic observation of the plasma membranes shows the preparation to consist mainly of smooth vesicles with a diameter of about 100–500 nm (Fig. 1). By means of electron microscopy, no nuclear or mitochondrial contamination is detectable.

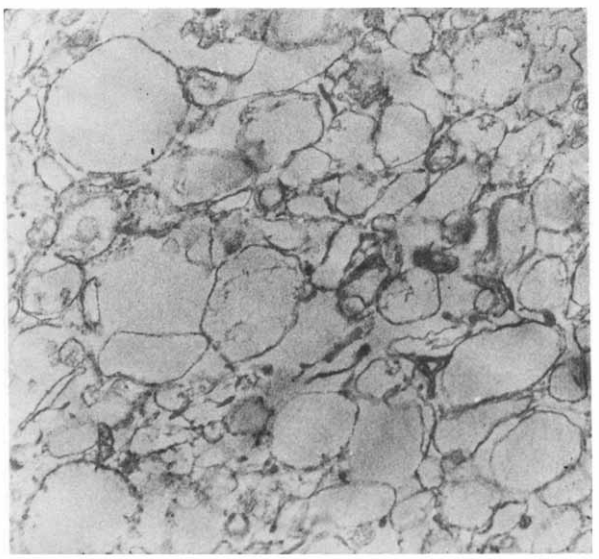

Fig. 1. Electron micrograph of plasma membrane vesicles. Plasma membranes were pelleted after gradient centrifugation as described under Materials and Methods. The pellet was fixed with glutaraldehyde, postfixed in OsO_4 and embedded in Epon. Thin sections were stained with uranyl acetate. The section was examined in a SEM 3/2 electron microscope of VEB Werk für Fernsehelektronik, GDR. Magnification ×30000.

As shown in Table I, the specific activity of $(Na^+ + K^+)$-ATPase is enriched about 20-fold over the homogenate, with a recovery of about 30%. Estimation of succinate dehydrogenase confirms the low degree of contamination of the plasma membrane preparation with membranes of mitochondrial origin.

Glucose-6-phosphatase activity as a marker for endoplasmic reticulum was too low for accurate determination in any cell fraction. Concluded from the sedimentation conditions of crude plasma membranes on differential centrifugation (3000 × *g*, 15 min), reticular contamination indeed seems very unlikely.

The specific activity of the lysosomal marker β-glucuronidase is enriched in the plasma membrane preparations compared with the homogenate. The lysosomal contamination is presumably due to the hypotonic lysis of cells. This contamination, however, should not have interfered with the investigations described in this paper.

TABLE I

DISTRIBUTION OF ENZYME ACTIVITIES IN VARIOUS FRACTIONS OF PLASMA MEMBRANE ISOLATION

The enzyme specific activities (S.A.) are expressed as mean values ($\mu mol \cdot h^{-1} \cdot mg^{-1}$) on the basis of the number of preparations given in the parentheses. All estimations were performed in duplicate. ($Ca^{2+} + Mg^{2+}$)-ATPase was assayed at 30°C, all other enzyme activities were estimated at 37°C. Rec., recovery (%).

Fraction	($Na^+ + K^+$)-ATPase		Mg^{2+}-ATPase [c]		($Ca^{2+} + Mg^{2+}$)-ATPase [d]		Succinate dehydrogenase		β-Glucuronidase	
	S.A.	Rec.	S.A.	Rec.	S.A.	Rec.	S.A.	Rec.	S.A.	Rec.
Homogenate	0.6 [a] (4)	100.0	2.0 [a] (4)	100.0	n.d.	n.d.	1.0 (4)	100.0	0.02 (4)	100.0
S 3000 × *g*	2.4 [a] (4)	28.8	2.9 [a] (4)	23.1	n.d.	n.d.	1.5 (4)	12.4	0.02 (4)	23.9
Plasma membranes	13.1 [a] (4)	29.9	5.6 [a] (4)	4.7	2.1 (5)	n.d.	0.9 (4)	1.2	0.09 (4)	6.5
	22.2 [b] (12)	n.d.	6.9 [b] (12)	n.d.						

[a] Estimation was performed in the presence of 1 mM deoxycholate.
[b] Estimation was performed after vesicle disruption by freezing and thawing.
[c] ATPase activity in the absence of sodium in the presence of K^+ and Mg^{2+}.
[d] ($Ca^{2+} + Mg^{2+}$)-ATPase was assayed under the ionic conditions of Ca^{2+} transport in isotonic sucrose medium.

We consider the intact plasma membrane vesicles to be mainly inside-out oriented, this being concluded from the following findings. The ($Na^+ + K^+$) – ATPase activity increases upon vesicle disruption by freezing and thawing (corresponding to an increased accesibility to ouabain of the ouabain-binding site at the extracellular surface, i.e., the intravesicular surface), while the total ATPase and ($Ca^{2+} + Mg^{2+}$)-ATPase activities remain unchanged after disruption of the vesicles (not shown), arguing for free accessibility to the cytoplasmic surface, i.e., the extravesicular surface, for ATP in the non-disrupted vesicles. Furthermore, the vesicles show a rapid Ca^{2+} accumulation (see below) after addition of ATP to the extravesicular medium as expected for the inside-out orientation of the plasma membrane with respect to its in vivo orientation.

Ca^{2+} transport and Ca^{2+}-dependent phosphorylation

As shown in Fig. 2, the Ca^{2+} transport into the vesicles in the absence of ATP is 5-times increased upon addition of ATP, as had also been described by us in the case of sarcolemmal membrane vesicles [10]. Ca^{2+} transport measurements with seven independent vesicle prepartaions yielded an average of 3.9 ± 1.3 nmol/mg protein for the ATP-dependent Ca^{2+} transport within a 20 min incubation.

Estimation of Ca^{2+} transport dependent on the ATP concentration, as demonstrated in the inset of Fig. 2, yielded a K_m (ATP) of 44 ± 9 μM ($n = 3$). Half-maximal activation of the ATP-dependent Ca^{2+} transport by Ca^{2+} is reached at 0.25 ± 0.10 μM ($n = 3$) (K_m (Ca^{2+})).

Preincubation of the membrane vesicles with 0.5 μM calmodulin from bovine brain at 30°C for 15 min had no effect on the ATP-dependent Ca^{2+} transport (not demonstrated).

Plasma membranes were phosphorylated with [γ-^{32}P]ATP at 0°C and subjected to an SDS-gel electrophoresis under acidic conditions.

The ionic conditions, especially the use of micromolar Mg^{2+} concentrations, were chosen to be similar to those successfully applied for identification of the ($Ca^{2+} + Mg^{2+}$)-ATPase of human erythrocytes [27]. The total amount of ^{32}P incorporated into the membranes in the presence of Ca^{2+} and micromolar Mg^{2+}, estimated by counting before electrophoresis, was in the range of 2–6 pmol/mg protein. A typical result from electrophoretic separation (the same principal patterns were obtained with five independent membrane preparations) is shown in Fig. 3b–d as an autoradiograph. The corresponding plasma membrane proteins have been visualized by staining with Coomassie blue (Fig. 3, lane a). In the presence of Ca^{2+} and micromolar Mg^{2+} (Fig. 3, lane b), three

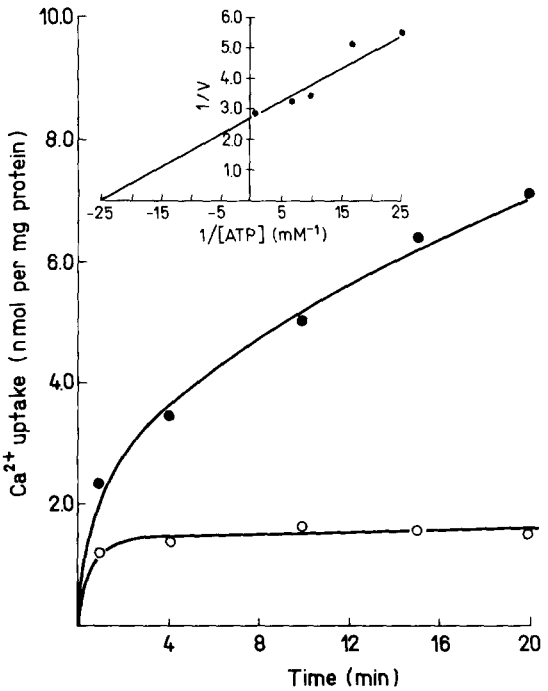

Fig. 2. ATP-effected change of Ca^{2+} accumulation into plasma membrane vesicles derived from Ehrlich ascites carcinoma cells. Plasma membrane vesicles were incubated with 5 mM $MgCl_2$ and 100 mM KCl in the presence (●) or absence (○) of 2 mM ATP as described under Matrials and Methods. The free Ca^{2+}-concentration was equal to 20 μM. Inset: Lineweaver-Burk plot of the dependence of initial rates of Ca^{2+} accumulation on ATP concentration. Initial rates were estimated as described above after 3 min of incubation time. The free Mg^{2+} concentration was equal to 3 mM for all ATP concentrations used.

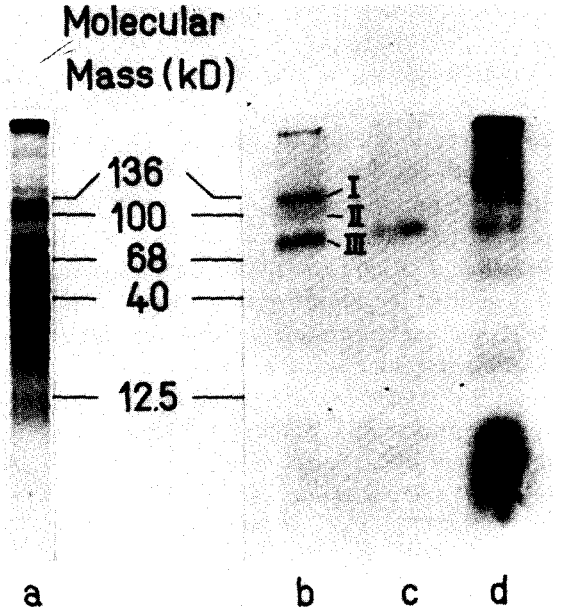

Fig. 3. Autoradiographic demonstration of the Ca^{2+}-dependent phosphoproteins in plasma membranes of Ehrlich ascites carcinoma cells. Plasma membrane proteins were separated on SDS-polyacrylamide gels as described under Materials and Methods and visualized by autoradiography after phosphorylation (lanes b–d, about 90 μg protein were applied per lane) or staining with Coomassie blue (lane a, about 40 μg protein were applied to the gel). Phosphorylation was performed with 2 μM [γ-^{32}P]ATP in the presence of 160 mM KCl, 12.5 μM $MgCl_2$ and 20 μM Ca^{2+} (lane b) or 160 mM KCl, 12.5 μM $MgCl_2$ and 500 μM EGTA (lane c) or 160 mM KCl, 5 mM $MgCl_2$ and 20 μM Ca^{2+} (lane d).

distinct bands are visualized, migrating with molecular masses of 135 (I), 100 (II) and about 90 kDa (III). Bands I and II disappear completely upon addition of EGTA into the phosphorylation medium (Fig. 3, lane c). Both bands are sensitive to hydroxylamine. Treatment with hydroxylamine decreased the total ^{32}P content of membranes from 100% (5.7 pmol ^{32}P per mg protein, control) to 15% (0.9 pmol ^{32}P per mg). Band III was independent of the presence of EGTA in the phosphorylation medium and insensitive to hydroxylamine treatment. Therefore, the identity of this band was not further investigated.

The strong dependence on Ca^{2+} and the acyl character of the protein-phosphate linkage of the dominant band I (Fig. 3, lane b) suggested its relation to the phosphorylated intermediate of the Ca^{2+} transport system.

The weakly visible acyl phosphate band II with a molecular mass of 100 kDa very likely corresponds to the phosphorylated intermediate of the ($Na^+ + K^+$)-ATPase of Ehrlich ascites cell plasma membranes (see below). The Ca^{2+}-dependent incorporation of radioactivity into this band can be explained by the findings of Schön et al. [28] that micromolecular Ca^{2+} is able to accelerate the Mg^{2+}-dependent phosphorylation reaction of ($Na^+ + K^+$)-ATPase. However, some contamination by the catalytic subunit of the ($Ca^{2+} + Mg^{2+}$)-ATPase of endoplasmic reticulum with its known molecular mass of 100 kDa cannot be excluded, although the sensitivity of this band to

Na^+ (see below) makes this interpretation unlikely.

Phosphorylation in the presence of millimolar Mg^{2+} revealed the same pattern of phosphoproteins in the high molecular mass range as found with the low Mg^{2+} concentration (Fig. 3, lane d). A number of additional phosphoproteins became detectable, presumably because of the activation of protein kinases by Mg^{2+} as described, for instance, for erythrocyte membranes [29]. A further increase in protein-kinase-dependent background phosphorylation in the presence of millimolar Mg^{2+} upon addition of EGTA (as deduced from measurements of protein kinase activity, unpublished data, Bartel et al.) rendered difficult any clear demonstration of the EGTA effect on the 135 kDa band in the presence of millimolar Mg^{2+}. The acyl phosphate character of phosphorylation of the 100 and the 135 kDa band was proven under these conditions as well (not shown).

The presence of millimolar Mg^{2+} permitted the clear identification of the 100 kDa protein as the catalytic subunit of the $(Na^+ + K^+)$-ATPase. As shown in Fig. 4, in the presence of Na^+, the ^{32}P incorporation into the 100 kDa peak is increased remarkably (Fig. 4a, b). This stimulation of the Mg^{2+}-dependent phosphorylation by sodium is known to be characteristic for the $(Na^+ + K^+)$-ATPase [30] and is demonstrated with a highly purified preparation of $(Na^+ + K^+)$-ATPase from pig kidney in Fig. 4c, d.

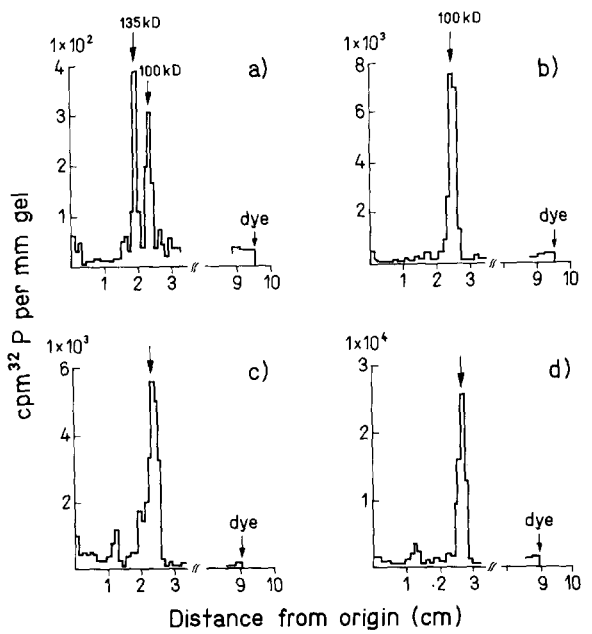

Fig. 4. Identification of the phosphoprotein of $(Na^+ + K^+)$-ATPase and differentiation from the phosphoprotein of $(Ca^{2+} + Mg^{2+})$-ATPase in plasma membranes of Ehrlich ascites carcinoma cells. Plasma membranes (Fig. 4a, b, 60 μg protein were applied per gel) or highly purified $(Na^+ + K^+)$-ATPase from pig kidney (Fig. 4c, d, 40 μg protein were applied per gel) were phosphorylated in the presence of 5 mM $MgCl_2$ and analyzed by SDS-gel electrophoresis, gel slicing and counting as described under Materials and Methods. Phosphorylation was performed either in presence of 20 μM Ca^{2+} (Fig. 4a, c) or in the absence of Ca^{2+} and in the presence of 140 mM NaCl and 1 mM EGTA (Fig. 4b, d) to demonstrate the effects of Ca^{2+} and Na^+ on ^{32}P incorporation into the different proteins.

Discussion

In the present paper we could demonstrate Ca^{2+}-dependent ATP splitting, ATP-dependent Ca^{2+} transport and the Ca^{2+}-dependent hydroxylamine-sensitive phosphorylation of a 135 kDa protein in highly purified plasma membranes from Ehrlich ascites carcinoma cells. We would like to suggest that the three phenomena are related to each other and are to be attributed to one and the same catalytic protein — the plasma membrane bound Ca^{2+}-transporting ATPase. This conclusion is supported by the following considerations:

Firstly, the molecular mass of the Ca^{2+}-dependent phosphorylated intermediate is clearly different from the known molecular mass of the very well investigated catalytic subunit of $(Ca^{2+} + Mg^{2+})$-ATPase of endoplasmic reticulum with a molecular mass of 100 kDA [8,9]. The 100 kDa phosphoprotein present in our preparations is attributed mainly to the catalytic subunit of $(Na^+ + K^+)$-ATPase as suggested strongly by its sensitivity to Na^+. On the other hand, as described, the plasma membranes are derived from a postnuclear low-speed pellet, while endoplasmic reticulum is known to sediment primarily in the microsomal fraction. Thus it is very unlikely that the observed characteristics of the Ca^{2+}-transporting ATPase should be caused by contamination of the plasma membrane preparation with endoplasmic reticulum.

Secondly, the formation of a phosphorylated intermediate dependent on the transported ion has

been shown to be characteristic for all cation-transporting ATPases. The molecular identities of $(Na^+ + K^+)$-ATPase [30], of the $(Ca^{2+} + Mg^{2+})$-ATPase of human erythrocyte membranes [27] as well as of the reticular $(Ca^{2+} + Mg^{2+})$-ATPase [8] have been established after the corresponding phosphorylated catalytic subunits had been demonstrated. Thus it seems to be a legitimate conclusion that the Ca^{2+}-dependent hydroxylamine-sensitive phosphorylation of a 135 kDa protein observed by us is related to Ca^{2+} transport and Ca^{2+}-dependent ATP-hydrolysis.

Finally, the characteristics of the enzyme described in the present paper are remarkably similar to those of the $(Ca^{2+} + Mg^{2+})$-ATPase of human erythrocyte membranes. The K_m values for ATP and Ca^{2+} were equal to 44 ± 9 μM or 0.25 ± 0.10 μM, respectively. The corresponding values for the $Ca^{2+} + MG^{2+}$)-ATPase of erythrocyte were found to be in the range of 25–60 μM [31,32] and 0.3–0.6 μM [32,33]. The molecular mass of the $(Ca^{2+} + Mg^{2+})$-ATPase of erythrocyte was estimated in the range of 140–150 kDa [33], while we have determined about 135 kDa for the Ca^{2+}-pump of Ehrlich ascites carcinoma cells. We could not, however, demonstrate any activation of Ca^{2+} transport by calmodulin as described for erythrocytes [33]. A calmodulin dependence of the Ca^{2+} pump of Ehrlich ascites cells cannot be entirely excluded, however, since we have not tried to deplete the plasma membranes of eventual endogenous calmodulin by EGTA treatment.

In recent years a number of reports on $(Ca^{2+} + Mg^{2+})$-ATPase activities and Ca^{2+}-transport properties of isolated plasma membranes of nucleated cells has appeared [4–7].

Hinnen et al. [34] have analyzed the Ca^{2+}-fluxes in Ehrlich ascites tumour cells. In plasma membrane preparations derived from these cells, which have not been characterized further, they detected a $(Ca^{2+} + Mg^{2+})$-ATPase activity of 50 nmol $\cdot$ $mg^{-1} \cdot min^{-1}$ at 37°C. Treating the membranes with phospholipids by the freeze-thaw sonication technique they obtained vesicles which exhibited an ATP-dependent Ca^{2+} accumulation with an efficiency similar to that which we obtained with native plasma membrane vesicles. The identity of the $(Ca^{2+} + Mg^{2+})$-ATPase was not further investigated. Several authors have dealt with attempts to identify phosphorylated intermediates of a putative plasma membrane bound $(Ca^{2+} + Mg^{2+})$-ATPase:

DeJonge et al. [35] and Schneider et al. [36] have published results of phosphorylation experiments with plasma membrane preparations from rat duodenal epithelium and macrophages, respectively. With macrophage plasma membranes [36], the authors suggested Ca^{2+}-dependent phosphorylation of a 132 kDa protein. Poor electrophoretic resolution and the absence of the 100 kDa peak of $(Na^+ + K^+)$-ATPase leaves some doubt, however, that the authors really have identified a Ca^{2+}-dependent phosphoprotein which is part of the plasma membrane. In the case of membranes from rat duodenal epithelium [35] a Ca^{2+}-dependent phosphoprotein is demonstrated as a poorly resolved shoulder of 115 kDa of a major Ca^{2+}-insensitive peak of 84 kDa. The authors claim [35] that the Ca^{2+}-sensitive band is co-migrating with ^{32}P-labelled $(Ca^{2+} + Mg^{2+})$-ATPase of sarcolemma membranes of rat heart. However, in native sarcolemma membranes, which are presumably always slightly contaminated by sarcoplasmic reticulum, we [10] and others [11] could not exclude that Ca^{2+}-dependent phosphorylation is due to the Ca^{2+}-pump of sarcoplasmic reticulum. Thus, the identity of the questionable 115 kDa phophoprotein shoulder described by DeJonge et al. [35] seems to be uncertain.

For the investigation of the sarcolemmal Ca^{2+} pump, contamination of sarcolemma membranes with membranes of sarcoplasmic reticulum origin represents the main problem. Thus, as mentioned before, our [10] previous findings and those of others [11] that in native sarcolemma membrane preparations the Ca^{2+}-dependent phosphorylation of only a 100 kDa protein is detectable cannot entirely be excluded to have been due to contamination of sarcolemma membranes with reticular membranes. Evidence for a Ca^{2+}-dependent phosphoprotein in sarcolemma membranes with a molecular mass (150 kDA) which is clearly different from that of the sarcoplasmatic Ca^{2+}-pump has been presented hitherto only by Caroni and Carafoli [12].

In contrast to results with sarcolemma membranes or plasma membranes from brain published by Caroni and Carafoli [37] and our-

selves [38], respectively, plasma membranes from Ehrlich ascites carcinoma cells, if checked in a manner similar to that described in Ref. 38 do not exhibit a Na^+-Ca^{2+} exchange (Eckert et al., unpublished data). Thus, for Ehrlich ascites cells the plasma membrane Ca^{2+}-pump identified seems to be the only possibility to realize an uphill Ca^{2+}-transport across te plasma membrane.

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