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THE PHOSPHOPROTEIN INTERMEDIATE OF A Ca^{2+} TRANSPORT ATPase IN RAT LIVER ENDOPLASMIC RETICULUM

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SUMMARY: Smooth endoplasmic reticulum vesicles from rat liver display an ATP-supported Ca^{2+} transport which is mediated by a (Ca^{2+} + Mg^{2+})-ATPase. During the catalytic cycle the terminal phosphate from ATP is incorporated to form an acid-precipitable reaction product(118 000-M_r in SDS-gel electrophoresis) with stability characteristics of an acylphosphate. Comparative studies with sarcoplasmic reticulum vesicles from fast-twitch skeletal muscle suggest that the 118 000-M_r phosphopeptide may be identified with the phosphorylated reaction intermediate of a Ca^{2+} transport ATPase in endoplasmic reticulum, similar to that in sarcoplasmic reticulum of muscle.

INTRODUCTION: An ATP-supported Ca^{2+} transport has been measured in rat liver microsomal fractions enriched with fragmented smooth and rough endoplasmic reticulum(1-3). It has been postulated that this active Ca^{2+} sequestration is linked to a Mg^{2+}-dependent and Ca^{2+}-stimulated ATPase intrinsic to the endoplasmic reticulum membrane(1). A certain similarity between the endoplasmic and sarcoplasmic reticulum Ca^{2+} transport systems has been suggested(1), although, direct evidence is lacking.

The present study describes a Ca^{2+} transport associated with endoplasmic reticulum fractions from rat liver, which is coupled to Ca^{2+}-stimulated ATP hydrolysis and formation of a trichloroaceticacid-precipitable phosphoprotein intermediate of acylphosphate nature with an app. molecular weight of 118 000. Some molecular and functional properties of this endoplasmic Ca^{2+} transport ATPase are compared to those of the Ca^{2+} pump in the sarcoplasmic reticulum of fast-twitch skeletal muscle.

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MATERIAL AND METHODS: Fractions enriched with smooth endoplasmic reticulum(ER)were prepared from the livers of male Wistar rats according to(4)using a gradient centrifugation on 1.3 M sucrose. Possible plasmalemmal and mitochondrial contamination was estimated by measuring specific activities of Na^+-/K^+-ATPase (5)and succinate dehydrogenase(6). Sarcoplasmic reticulum vesicles(SR)were isolated from the white portion of rabbit psoas muscle as described(7).

ATPase- and Ca^{2+} uptake measurements: Mg^{2+}-ATPase activity was determined for 30 min at 25°C in a medium containing: 42.7 mM Hepes-KOH buffer(pH 7.0), 0.1 M KCl, 5 mM $MgCl_2$, 5 mM NaN_3, 2 mM Tris-EGTA, 5 mM Tris-ATP and 0.467 mg/ml ER vesicles. The liberated P_i was determined according to (8). (Ca^{2+} + Mg^{2+})-ATPase activity was measured in the same medium replacing Tris-EGTA by 50 µM $CaCl_2$. Ca^{2+}-ATPase activity was expressed as the difference between (Ca^{2+} + Mg^{2+})-ATPase and Mg^{2+}-ATPase according to (9). Ca^{2+} uptake was measured as described(7, 10)at 25°C in the following medium: 45.4 mM Hepes-KOH buffer(pH 6.8), 66.6 mM KCl, 5 mM $MgCl_2$, 11.2 µM $CaCl_2$ mixed with $^{45}CaCl_2$, 5 mM potassium oxalate, 5 mM NaN_3, 5 mM Tris-ATP and 0.172 mg/ml ER. Control measurements were performed replacing Tris-ATP by Hepes-KOH buffer. Ca^{2+} uptake measurements in the absence of oxalate were carried out with 0.502 mg ER/ml assay mixture.

Phosphoprotein formation and analyses of phosphorylated compounds: ER and SR vesicles were phosphorylated as has been described(7, 10). Incubations lasted 20 s at 0°C in 0.5 ml mixtures of 38.4 mM Hepes-KOH buffer(pH 6.8), 0.1 M KCl, 5 mM NaN_3, 3.33 nM [γ-^{32}P]ATP(specific radioactivity 6000 Ci/mmol)and 0.5 mg protein. Modifications of the ionic conditions are indicated in the legends to the table and figures. Electrophoreses were performed according to Laemmli(11)and Weber and Osborn(12). Phosphopeptides were visualized radioautographically(7, 10).

Protein determination: The method of Lowry et al.(13)was used with human serum albumin for calibration.

Chemicals: All chemicals were of the highest purity available. [γ-^{32}P]ATP was from The Radiochemical Centre(Amersham, England).

RESULTS AND DISCUSSION: Fig. 1 shows the time course of Ca^{2+} uptake by smooth ER vesicles. Addition of oxalate enhanced both, velocity and capacity of Ca^{2+} transport. Accumulated Ca^{2+} could be readily released from the vesicles by adding the ionophore a 23187(Fig. 1). Ca^{2+} binding in the absence of ATP(oxalate present)(Fig. 1)is very low and reached about 1.2 nmol Ca^{2+}/mg protein x 15 min. Activities of ATPases and Ca^{2+} uptake are given in table 1. The Ca^{2+}-ATPase activity is low, corresponding to the low Ca^{2+} pumping activity. Because of the very low activity of Ca^{2+}-ATPase we were unable to determine the initial rate with sufficient accuracy. Therefore, ATPase activities in table 1 refer to 30 min incubations. Azide did not inhibit either Ca^{2+}-ATPase or Mg^{2+}-ATPase.

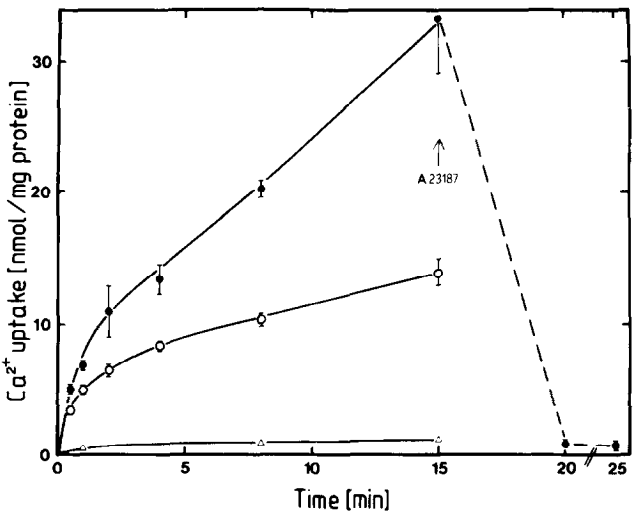

Fig. 1. Time course of Ca^{2+} uptake by ER vesicles from rat liver. Incubations were performed in the presence(●-●)and in the absence(Δ-Δ)of ATP and without oxalate(o-o). After 15 min of incubation the ionophore A 23187 was added at a final concentration of 10 µM.

Qualitative analysis of fractions enriched with smooth ER vesicles, which were phosphorylated with [γ-^{32}P] ATP, was performed by means of sodium dodecylsulfate polyacrylamide gel electrophoresis followed by radioautography. Phosphorylation in the presence of Mg^{2+} and EGTA produced several phosphopeptides with app. molecular weights(M_r)of 122 000 - 130 000, 45 000, 42 500, 23 000 and 12 000(Fig. 2 a). Phosphopeptides of 173 000-, 145 000-, 130 000-, 120 000-, 60 000 - 56 000-, 49 000 - 51 000- and 17 000-M_r have been described(14-17). The 145 000- and

Table 1

Activities of ATPases and Ca^{2+} uptake in ER vesicles from rat liver.

Mg^{2+}-ATPase	Ca^{2+}-ATPase	Ca^{2+} uptake	
		with oxalate	without oxalate
µmol P_i x 30 min^{-1} x mg $protein^{-1}$		nmol x min^{-1} x mg $protein^{-1}$	
1.287 ± 0.026(n=9)	0.068 ± 0.041(n=9)	6.87 ± 0.18(n=4)	4.48 ± 0.10(n=4)

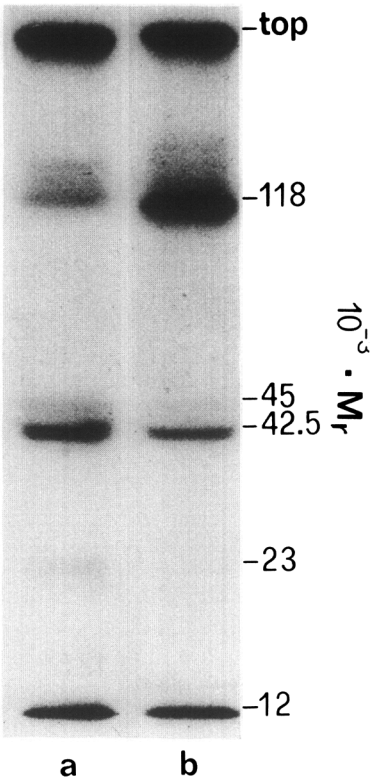

Fig. 2. Radioautographs of phosphopeptide patterns in ER vesicles from rat liver. Phosphorylation lasted 20 s at 0°C in the presence of: (a) 6 mM $MgCl_2$ + 1 mM EGTA; (b) 6 mM $MgCl_2$ + 50 µM $CaCl_2$. Electrophoreses were carried out according to Laemmli(11) with 60 µg protein, respectively.

130 000-M_r peptides have been identified by Lam and Kasper(17)to represent substrates for a membrane bound pyrophosphate:protein phosphotransferase. Phosphorylation of these peptides was inhibited by Ca^{2+}(16). The nature of the other Mg^{2+}-dependent phosphorylated peptides and the phosphorylated material near the top of the gel(Fig. 2)is unknown. If Ca^{2+} is additionally included into the incubation mixture significant ^{32}P incorporation occurs into a peptide of 118 000-M_r(Fig. 2 b). A 115 000-M_r phosphopeptide has been identified with the phosphorylated reaction intermediate of the Ca^{2+} transport ATPase within the SR membrane(7, 10, 18). The small differences in the app. molecular weights of the Ca^{2+}-dependent phosphorylated 100 000-M_r peptides in SR and ER mem-

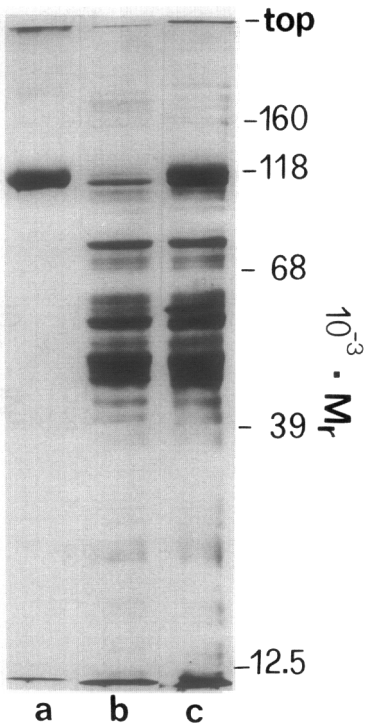

Fig. 3. Electrophoreses of SR vesicles from fast-twitch muscle(a) and ER vesicles from rat liver(b). 5 µg SR(a) and 20 µg ER(b) were applied. c shows a co-electrophoresis of 3 µg SR with 20 µg ER. The method of Laemmli was used(11).

branes may be explained by the different protein concentrations, which influence the electrophoretic mobility. The patterns in Fig. 3 show that the SR Ca^{2+} transport ATPase(Fig. 3 a) amounts a high percentage of total membrane protein, while the 118 000-M_r peptide represents only a very small fraction of the ER(Fig. 3 b).

Ca^{2+}-stimulated ^{32}P incorporation into the 118 000-M_r peptide was further documented by phosphorylation in the presence of different Ca^{2+} concentrations. Fig. 4 shows that increasing amounts of ^{32}P were incorporated into the 118 000-M_r peptide, in parallel with increasing concentrations of Ca^{2+}.

It is well known that ^{32}P incorporation into the SR Ca^{2+} transport ATPase occurs at an aspartyl residue. The resulting

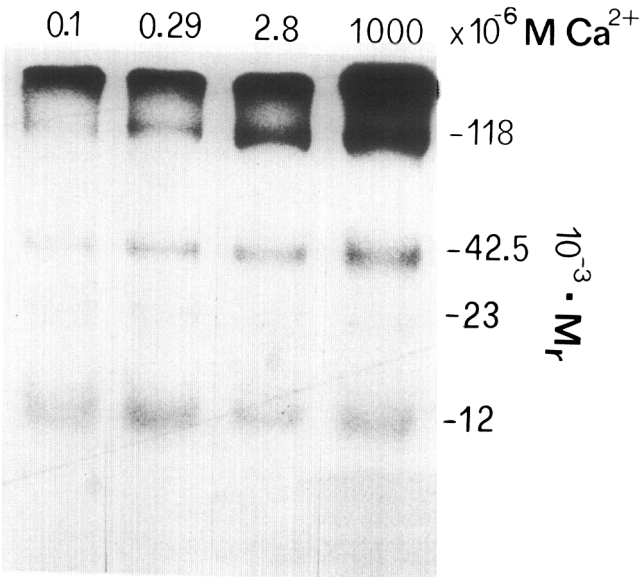

Fig. 4. Radioautographs of SDS-gel-electrophoreses from ER vesicles, phosphorylated at different concentrations of free Ca^{2+}. Phosphorylation was performed at 0°C in the presence of 38.4 mM Hepes buffer(pH 6.8), 0.1 M KCl, 5 mM NaN_3, 6 mM $MgCl_2$, 3.33 nM [γ-^{32}P] ATP, 0.5 mg/ml protein and various concentrations of free Ca^{2+}, which were defined using an apparent stability constant for Ca-EGTA of $10^{5.94}$(19). Electrophoreses were carried out according to Weber and osborn(12)with 50 μg protein applied to the gel.

acylphosphate is unstable in the presence of hydroxylamine and undergoes rapid decomposition(20), while phosphoesters remain stable. This phenomenon allows us to distinguish acylphosphates from alkylphosphates in phosphorylated SR membranes(20, 21). Fig. 5 A depicts the time-dependent hydroxylamine-induced decomposition of acylphosphate in liver fractions enriched with smooth ER vesicles. The time course of decomposition resembles that observed with phosphorylated SR vesicles from fast-twitch skeletal muscle(Fig. 5 B). From the radioautographs(inset Fig. 5 A)it may be seen that breakdown of acylphosphate occurs in the 118 000-M_r phosphopeptide, while the Mg^{2+}-dependent formed phosphopeptides are hydroxylamine-resistent. The 118 000-M_r phosphopeptide cannot be explained by contaminants as glycogen phosphorylase a or autocatalytically phosphorylated α and β

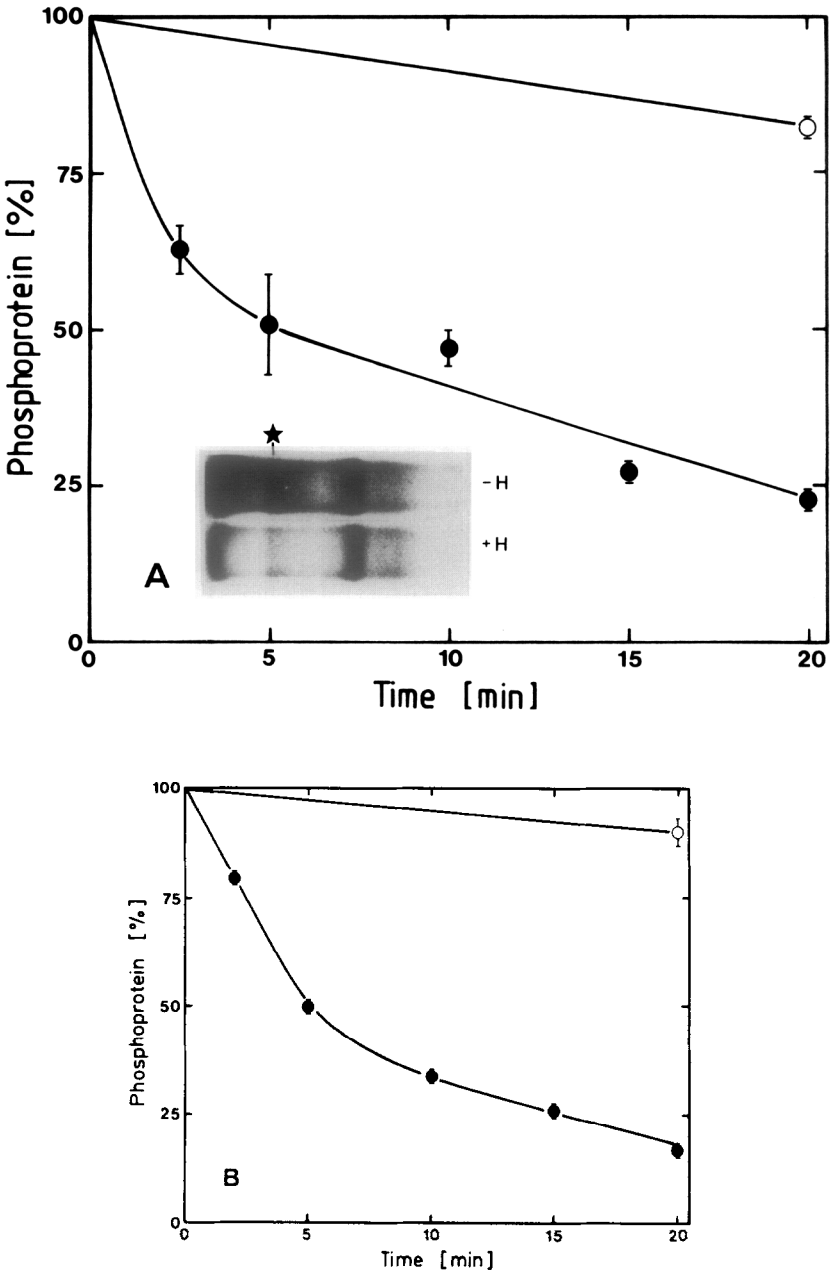

Fig. 5. Effect of hydroxylamine on acid-denatured phosphoproteins of ER from rat liver(A)and SR from fast-twitch skeletal muscle(B). Phosphorylation was performed as in Fig. 2 with 50 μM $CaCl_2$ added. Precipitated and extensively washed protein was suspended in sodium acetate buffer(pH 5.2)(o-o)or 0.8 M hydroxylamine in sodium acetate buffer(pH 5.2)(•-•)and incubated at 5°C. Reactions were terminated with 10% TCA. The inset shows radioautographs of electrophoreses(12)from phosphorylated ER vesicles incubated for 20 min with(+H)and without(-H) hydroxylamine. The asterix indicates the 118 000-M_r phosphopeptide.

Table 2

Phosphoprotein formation in ER vesicles in different ionic milieu at low ATP concentration. Incubations were performed as described in Fig. 2.

Conditions	^{32}P incorporation (pmol x 20 s^{-1} x mg $protein^{-1}$)
6 mM $MgCl_2$ + 1 mM EGTA	0.102 ± 0.003
6 mM $MgCl_2$ + 50 μM $CaCl_2$	0.278 ± 0.004

subunits of phosphorylase kinase, since the latter form alkylphosphates. As shown recently(22), alkylphosphate formation may occur in the SR Ca^{2+} transport ATPase. The pssibility that the 118 000-M_r peptide may also incorporate a very small amount of alkylphosphate cannot be excluded.

Our qualitative analyses of phosphoprotein formation are supported by quantitative measurements. The data in table 2 indicate Ca^{2+}-stimulated ^{32}P incorporation into ER membranes.

These results led us to the following conclusions. ER vesicles from rat liver display an ATP-supported Ca^{2+} uptake. During the catalytic cycle ^{32}P from $[\gamma\text{-}^{32}P]$ ATP is incorporated to form a reaction product with stability characteristics of an acylphosphate. ^{32}P incorporation occurs into a 118 000-M_r peptide and requires μM Ca^{2+}. A comparison with SR vesicles from fast-twitch skeletal muscle revealed qualitative similarities between the 115 000-M_r Ca^{2+} transport ATPase and the 118 000-M_r peptide from ER, i. e. similar M_r and Ca^{2+}-dependent formation of an acid-precipitable acylphosphate. Therefore, the phosphorylated 118 000-M_r peptide may be identified with the phosphorylated reaction intermediate of a Ca^{2+} transport ATPase in ER of rat liver, analogous to that in SR of muscle.

The quantitative calculations were made on a protein basis. Therefore, the specific catalytic activities for Ca^{2+} uptake, ATPases and phosphoprotein formation may be underestimated, since the 118 000-M_r peptide represents only a minor fraction of total endoplasmic reticulum membrane protein.

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