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Chimeric Ca^{2+}-ATPase/Na^+,K^+-ATPase molecules

Their phosphoenzyme intermediates and sensitivity to Ca^{2+} and thapsigargin

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Chimeric molecules consisting of parts from the sarcoplasmic reticulum Ca^{2+}-ATPase and the Na^+,K^+-ATPase were expressed in COS-1 cells and analysed functionally. One chimera, in which most of the central cytoplasmic loop was derived from the Na^+,K^+-ATPase, while the transmembrane segments and the minor cytoplasmic loop came from the Ca^{2+}-ATPase, was able to occlude Ca^{2+} and to be phosphorylated from ATP with normal apparent affinity for Ca^{2+} and ATP. This chimera also displayed normal sensitivity to thapsigargin, but was unable to undergo the transition from ADP-sensitive to ADP-insensitive phosphoenzyme and to transport Ca^{2+}. The other chimera, which consisted of the NH_2-terminal two-thirds of Na^+,K^+-ATPase and the COOH-terminal one-third of Ca^{2+}-ATPase, was unable to phosphorylate from ATP, but phosphorylated from inorganic phosphate in a Ca^{2+}-inhibitable and thapsigargin-insensitive reaction. These results can be explained in terms of a structural model in which the non-conserved residues in the central cytoplasmic domain of the Ca^{2+}-ATPase are without major importance for the binding and occlusion of Ca^{2+}, but are involved in the E1P→E2P conformational changes of the phosphoenzyme, whereas residues in transmembrane segments on both sides of the central cytoplasmic domain are involved in formation of the Ca^{2+}-binding sites. The data moreover show that thapsigargin sensitivity is dependent on residues in the NH_2-terminal one-third of the Ca^{2+}-ATPase molecule.

Ca^{2+} occlusion; Ca^{2+}-pump; Chimera; Na^+,K^+-pump; Thapsigargin

1. INTRODUCTION

The Ca^{2+}-ATPase of sarcoplasmic reticulum (SR) and the Na^+,K^+-ATPase of mammalian plasma membranes both belong to the P-type family of membrane bound cation transporting ATPases. Detailed amino acid sequence comparisons have shown that it is possible to align the sequences of P-type ATPases with different cation selectivity [1], and although the existence of a consensus structure for P-type ATPases has only in part been confirmed by more direct methods, it seems clear that the basic structural elements of these ATPases are similar, consisting of two cytoplasmic domains anchored to a membrane sector that most likely is made up from an even number (8 or 10) of transmembrane α-helices. A central issue in the elucidation of structure-function relationships in the P-type ATPases is the location of the binding sites for the transported cations and ATP [2–4]. The understanding of the transport mechanism would in addition be furthered by identification of residues involved in the binding of specific inhibitors that prevent cation binding or conformational changes. One such inhibitor is thapsigargin, which acts specifically on the sarco(endo)plasmic reticulum Ca^{2+}-ATPases to reduce the Ca^{2+} affinity [5,6].

The use of site-directed mutagenesis has provided a wealth of information regarding the functional roles of single amino acid residues in the sarcoplasmic reticulum Ca^{2+}-ATPase. Hence, these studies have identified residues of importance for Ca^{2+} binding [7,8], ATP binding and phosphorylation [3,9], and E1P→E2P [10–12] as well as E2P→E2 [12–14] conformational changes. All residues found to be crucial to ATP binding and phosphorylation are located in the central cytoplasmic domain, whereas residues that appear to be involved in Ca^{2+} binding are located in the putative transmembrane segments [3]. This points to a physical separation of 'phosphorylation' and 'Ca^{2+}-binding' domains, which seems to be supported also by the use of proteolytic degradation methods [4]. Somewhat surprisingly, most of the residues with a potential role as Ca^{2+} ligands are highly conserved among the P-type ATPases [1,2,7]. Thus, the variable residues that directly or indirectly determine the cation selectivity of the ATPases remain to be identified. Information is also lacking concerning

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Abbreviations: Ca^{2+}-ATPase, Ca^{2+}-activated adenosine triphosphatase; $C_{12}E_8$, octaethyleneglycol monododecyl ether; E1P, ADP-sensitive phosphoenzyme intermediate; E2P, ADP-insensitive phosphoenzyme intermediate; EGTA, [ethylenebis(oxyethylene-nitrilo)] tetraacetic acid; M1-M10, putative transmembrane segments numbered from the NH_2-terminal end of the peptide; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propane- sulfonic acid; Na^+,K^+-ATPase, Na^+- and K^+-activated adenosine triphosphatase; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; SDS, sodium dodecyl sulfate; SR, sarcoplasmic reticulum.

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the roles of the large number of non-conserved residues present in the central cytoplasmic domain.

To address these problems it should be useful to study chimeric constructs, in which whole peptide segments have been swopped between ATPases of different cation selectivity. The basic similarity between the overall structures of P-type ATPases deduced from the amino acid sequences seems to provide a sound theoretical basis for the construction of such chimeric ATPases. In principle, studies of chimeric molecules should make it feasible to evaluate the functional significance of several substitutions of non-conserved residues and also to test the current ideas regarding domain structure and boundaries. If the assumption of a common structure of the various P-type ATPases holds, and the ATP and cation binding domains are separate, it might be feasible to exchange the ATP binding domains without change of cation selectivity.

Recently, functional chimeric P-type ATPases have been produced, although the information on their enzymatic properties was limited by low expression levels [15,16]. In the present study, we have constructed two chimeric molecules consisting of parts of Ca^{2+}-ATPase and Na^+,K^+-ATPase (Fig. 1), and we have taken advantage of the relatively high expression efficiency obtained with the COS-1 cell system to carry out a detailed functional analysis of these chimeric ATPases, using phosphorylation and occlusion assays developed previously for analysis of point mutants [8,10,11].

2. MATERIALS AND METHODS

To construct chimeric cDNAs by exchange of cDNA fragments between the rabbit fast twitch muscle Ca^{2+}-ATPase (SERCA1a) cDNA [9] and the rat kidney $\alpha 1$ Na^+,K^+-ATPase cDNA [17], oligonucleotide-directed mutagenesis [18] was used to introduce unique restriction sites in highly conserved areas (Fig. 1) without changing the amino acid sequences encoded by the cDNAs. One site (*NdeI*) was introduced in the cDNA sequence encoding amino acid residues 348–349, the other (*HpaI*) was introduced in the cDNA sequence encoding amino acid residues 705–706 (numbering referring to the SR Ca^{2+}-ATPase). The presence of the correct mutation was confirmed by nucleotide sequencing by the dideoxynucleotide chain termination method with modifications as described [11].

The full-length Ca^{2+}-ATPase/Na^+,K^+-ATPase chimeric cDNAs were cloned into the expression vector pMT2 [19], and the cesium chloride gradient-purified plasmids were transfected into COS-1 cells by the calcium phosphate procedure [17]. Microsomes were prepared from the cells 48–60 h after the transfection as described in [9]. In some experiments we used an alternative preparation procedure described in [17] to obtain a high yield of plasma membrane derived microsomes.

Immunoblotting following SDS-polyacrylamide gel electrophoresis was carried out as in [11] using monospecific anti-Ca^{2+}-ATPase antibodies raised against synthetic peptides [20]. Ca^{2+}-activated ATP-driven Ca^{2+} transport was assayed by Millipore filtration [11]. Phosphorylation from [γ-^{32}P]ATP or $^{32}P_i$ and the ADP-sensitivity of the phosphoenzymes was measured as previously [10,11,13] with modifications as described in the figure legends. To study thapsigargin-inhibition of phosphorylation, the microsomes were preincubated for 30 min with the indicated concentration of thapsigargin. All phosphorylation experiments were performed at least two times with closely agreeing results. After acid precipitation, the phosphorylated microsomal protein was washed and subjected to SDS-polyacrylamide gel electrophoresis at pH 6.0 followed by autoradiography of the dried gels and quantitation by densitometric analysis using an LKB 2202 Ultroscan Laser Densitometer.

Ca^{2+} occlusion was measured essentially as described in [8]. In brief, the isolated microsomal membranes were incubated for 4 h at 20°C in 300 μl of a reaction mixture containing 10 μM $^{45}Ca^{2+}$, 1 mM CrATP, 5 mM Mg^{2+}, 0.1 M NaCl, and 50 mM TES/Tris buffer (pH 7.0). Following this incubation, the membranes were solubilized by addition of the non-ionic detergent $C_{12}E_8$ at a concentration of 5 mg/ml. Insoluble material was removed by centrifugation for 30 min in a Beckman Airfuge at 130,000 × g, and 250 μl of the supernatant was subjected to molecular sieve HPLC in a TSK G 4000 SW column (TosoHaas) in the presence of 1.5 mM non-radioactive Ca^{2+} in the eluant. The collected fractions were analysed for radioactivity by liquid scintillation counting and for Ca^{2+}-ATPase content by immunoblotting as described above.

3. RESULTS

The cDNAs encoding the chimeric fusion proteins shown in Fig. 1 were expressed transiently in transfected COS-1 cells. In Chimera A, most of the largest cytoplasmic loop is derived from the Na^+,K^+-ATPase, while the transmembrane segments and the minor cytoplasmic loop comes from the Ca^{2+}-ATPase. Chimera B consists of the NH_2-terminal two thirds of Na^+,K^+-ATPase and the COOH-terminal one third of Ca^{2+}-ATPase. Thus, in both cases the expression could be analysed using monospecific antibodies against characteristic Ca^{2+}-ATPase sequences in the COOH-terminal one third of the molecule. Since the Na^+,K^+-ATPase is transported to the plasma membrane and the SR Ca^{2+}-ATPase to the endoplasmic reticulum, we used two different methods for preparation of membrane fractions. One method [9] led to microsomes with a reduced plasma membrane content, whereas the other method [17] resulted in a preparation enriched with plasma membrane. For as well Chimera A as Chimera B, the former preparation procedure gave the highest yield as judged from immunoreactivity, indicating that the chimeric proteins were retained predominantly in the endoplasmic reticulum, and this preparation procedure was subsequently used.

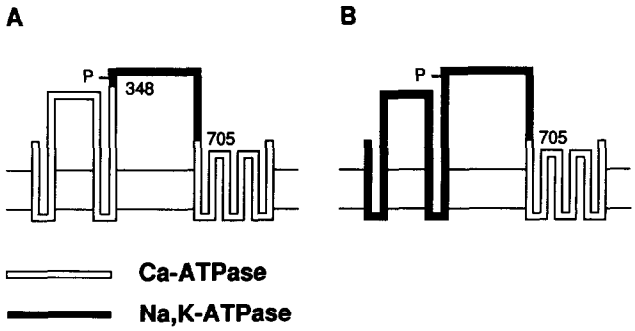

Fig. 1. Schematic representation of the chimeric fusion proteins examined in the present study. The 'P' indicates the phosphorylated aspartic acid residue. Numbers locate points of recombination (referring to the Ca^{2+}-ATPase).

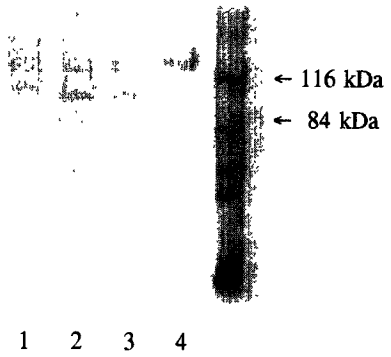

Fig. 2. Examination of expression by immunoblotting. Microsomes from COS-1 cells transfected with either wild-type Ca^{2+}-ATPase cDNA (lane 1), cDNA encoding Chimera A (lane 2) or Chimera B (lane 3), or microsomes from cells that had been mock-transfected with the vector without insert (lane 4), were subjected to SDS-polyacrylamide gel electrophoresis followed by transfer to nitrocellulose. Equal amounts of total microsomal protein were loaded in the lanes. The blot was developed using antibody raised against the sequence GFNPPDLDIMD (residues 808–818 in SR Ca^{2+}-ATPase) in combination with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. The arrows indicate molecular weight standards.

Fig. 2 shows an example of an immunoblot of expressed wild-type Ca^{2+}-ATPase and the chimeric ATPases, which had been subjected to SDS-polyacrylamide gel electrophoresis. In this experiment, an antibody raised against a peptide corresponding to residues 808–818 of the SR Ca^{2+}-ATPase [20] was used to visualize the protein. In addition to the expressed wild-type Ca^{2+}-ATPase and the chimeric ATPases, which migrated corresponding to molecular weights between 116,000 and 84,000, an endogenous COS-cell protein with apparent molecular weight close to 140,000 is seen to react with the antibody. The chimeric proteins did not migrate corresponding to exactly the same relative molecular mass as the wild-type Ca^{2+}-ATPase. Presumably, this was due to the difference in the number and charge of the residues (wild-type Ca^{2+}-ATPase: 994 residues, 27 net negative charges; Chimera A: 983 residues, 36 net negative charges; Chimera B: 1003 residues, 36 net negative charges).

Quantitation of expression levels by comparison with known amounts of Ca^{2+}-ATPase on immunoblots similar to that shown in Fig. 2 indicated that the yield of Chimera A varied between 70% and 150% that of the wild type, while the yield of Chimera B varied between 10% and 45% that of the wild type, in 5 transfections.

The Ca^{2+} transport activities of the chimeric molecules and the wild-type Ca^{2+}-ATPase were examined at Ca^{2+} concentrations up to 50 μM using a filtration assay following 10 min incubation of the microsomes with $^{45}Ca^{2+}$ and 5 mM MgATP at 27 °C [11]. In Chimera A as well as Chimera B, the Ca^{2+} transport rate was immeasurably low, i.e. < 5% that of the wild-type Ca^{2+}-ATPase. In Chimera A, Ca^{2+} binding at high affinity sites could nevertheless be demonstrated by the ability of this chimera to occlude Ca^{2+} in the presence of CrATP. This is seen in Fig. 3, which shows the measurement of occluded Ca^{2+} by HPLC in a TSK G 4000 SW size exclusion column (cf. [8] and [21]). The chimeric protein eluted together with a peak of radioactive $^{45}Ca^{2+}$, which was absent from the control sample containing microsomes from COS cells mock-transfected with the expression vector without insert. Experiments with wild-type Ca^{2+}-ATPase showed a peak of occluded Ca^{2+} of the same magnitude as that observed for Chimera A (not shown, but see [8]).

The ability of Chimera A to occlude Ca^{2+} not only demonstrated that the Ca^{2+} binding sites functioned, but to allow CrATP to stabilize Ca^{2+} occlusion the ATP binding site must also have been relatively intact in this chimera. It should be noted that in the normal reaction cycle the binding of Ca^{2+} and ATP is followed by transfer of the terminal phosphoryl group of ATP to the enzyme. This reaction is, however, not required for Ca^{2+} occlusion supported by CrATP [8,21].

The abilities of the chimeric ATPases to phosphorylate were tested using radioactive [γ-^{32}P]ATP and $^{32}P_i$. We found that Chimera A was able to form an acid-stable phosphoenzyme with a steady-state concentration similar to that of the wild-type Ca^{2+}-ATPase in the presence of 2 μM [γ-^{32}P]ATP and micromolar concen-

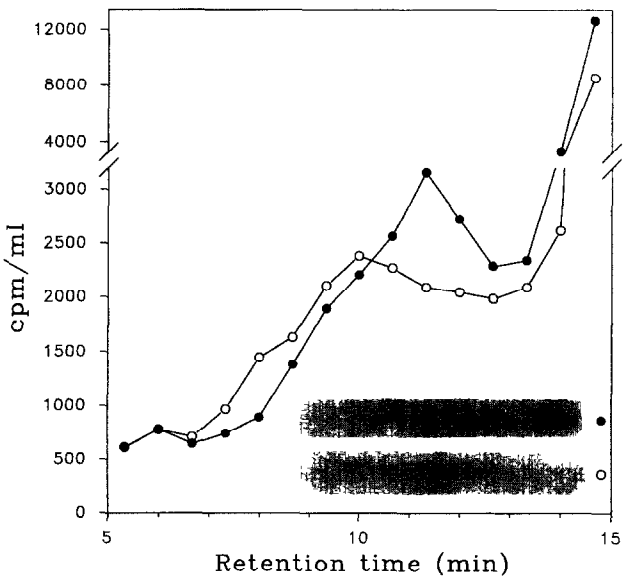

Fig. 3. Measurement of CrATP-supported Ca^{2+} occlusion in Chimera A. The Ca^{2+}-occluded enzyme was formed by incubation of the microsomal membranes in the presence of 10 μM $^{45}Ca^{2+}$ and CrATP as described in Section 2. Following centrifugation to remove insoluble material 250 μl sample was injected into the HPLC column. The eluant contained 5 mg $C_{12}E_8$/ml, 0.1 M NaCl, 50 mM TES (pH 7.0), 1.5 mM $^{40}CaCl_2$, 1.0 mM EGTA, and 10 mM $MgCl_2$. Fractions of 0.5 ml were collected for analysis of radioactivity (ordinate) or immunoreactivity by the blotting technique (inserts). Microsomes were harvested from COS-1 cells transfected with cDNA encoding Chimera A (●) or from cells transfected with the expression vector without insert (○). The blots shown as inserts are aligned so that the lanes are right below the corresponding points.

trations of Ca^{2+}. In contrast, Chimera B was unable to form any detectable phosphoenzyme under these conditions. Neither did increased levels of Ca^{2+} (up to 10 mM) and of ATP (10 μM) induce phosphoenzyme formation in Chimera B, and substitution of Na^+ (100 mM) for K^+ in the reaction buffer was also without effect (not shown), although two thirds of Chimera B is derived from the Na^+,K^+-ATPase, and Na^+ activates phosphorylation from ATP in the wild-type Na^+,K^+-ATPase. In Chimera A, the presence of 100 mM Na^+ in the absence of Ca^{2+} and K^+ was likewise unable to activate phosphorylation from ATP.

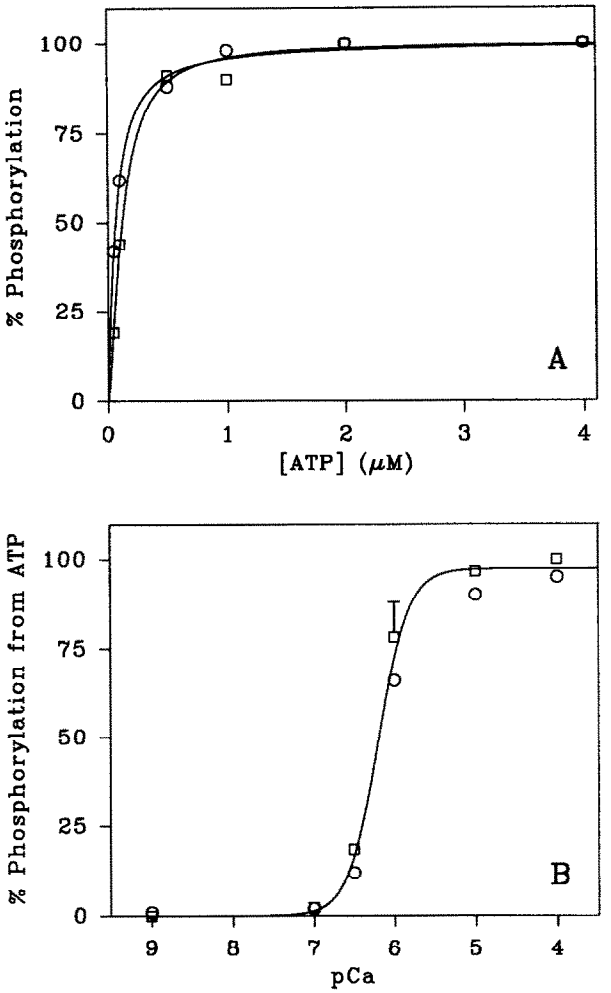

Fig. 4. ATP and Ca^{2+} dependencies of phosphorylation from ATP of wild-type Ca^{2+}-ATPase and Chimera A. Phosphorylation of the microsomal fractions isolated from COS-1 cells transfected with cDNA encoding either wild-type Ca^{2+}-ATPase (□) or Chimera A (○) was carried out at 0 °C for 15 s in the presence of 20 mM MOPS buffer (pH 7.0), 80 mM K^+, 5 mM Mg^{2+} and various concentrations of [γ-^{32}P]ATP and Ca^{2+}. (A) Varying [ATP] and constant [Ca^{2+}] (100 μM). (B) varying [Ca^{2+}] and constant [ATP] (2 μM). The acid-quenched ^{32}P-phosphorylated samples were subjected to SDS-polyacrylamide gel electrophoresis at pH 6.0, and the autoradiograms of the dried gels were quantitated by densitometry. The specific phosphorylations are shown as percentages of their maximal values. Mean values of duplicate determinations are shown with the error bar indicating the largest deviation from the mean.

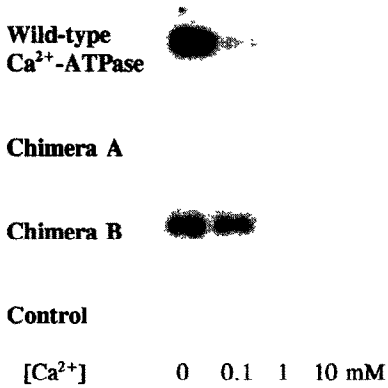

Fig. 5. Ca^{2+} dependency of phosphorylation from P_i. Phosphorylation of microsomes isolated from COS-1 cells transfected with cDNA encoding either wild-type Ca^{2+}-ATPase, Chimera A, or Chimera B, or of microsomes isolated from cells transfected with vector without insert ('Control') was carried out at 25 °C for 10 min in the presence of 100 mM MES/Tris buffer (pH 6.0), 5 mM Mg^{2+}, 30% (v/v) dimethylsulfoxide, 500 μM [^{32}P]P_i, and 2 mM EGTA ('0') or the indicated Ca^{2+} concentrations. The acid-quenched ^{32}P-phosphorylated samples were subjected to SDS-polyacrylamide gel electrophoresis at pH 6.0, and the autoradiograms of the dried gels are shown.

The ATP-concentration dependencies of phosphorylation of wild-type Ca^{2+}-ATPase and Chimera A were compared by varying the ATP-concentration between 0.05 μM and 4 μM at a constant (saturating) Ca^{2+} concentration of 100 μM. As seen in Fig. 4A, the apparent affinity for ATP displayed by Chimera A was identical to or perhaps even slightly higher than that of the wild-type Ca^{2+}-ATPase. The effect of Ca^{2+} concentration on formation of phosphoenzyme from ATP was analysed in a similar way, and no difference was detected between the wild type and Chimera A (Fig. 4B). Since the binding of two calcium ions is required for activation of the transfer of the γ-phosphoryl group of ATP to the enzyme [22], it may be deduced that either Ca^{2+} site was intact in Chimera A, as also indicated by the occlusion experiment. Thus, it seems that Chimera A retained the full capability to react with ATP and Ca^{2+}, but somehow was unable to proceed the reaction cycle and transport Ca^{2+}.

Both the Ca^{2+}-ATPase and the Na^+,K^+-ATPase can be phosphorylated 'back door' by inorganic phosphate (P_i), when the enzymes are in the E2 conformation. Fig. 5, first lane ([Ca^{2+}] = '0'), shows the result of an experiment in which the abilities of the chimeric ATPases to phosphorylate from P_i were examined under optimum conditions for stabilization of the E2/E2P forms, in the absence of Ca^{2+}, Na^+, and K^+, and in the presence of the organic solvent dimethylsulfoxide, which is known to promote the binding of P_i, possibly due to an effect on the structure of water [23]. The level of phosphorylation from P_i was very low in Chimera A and could hardly be distinguished from the background. By contrast, Chimera B phosphorylated from P_i. Although, as seen in Fig. 5, the amount of phosphoprotein was lower

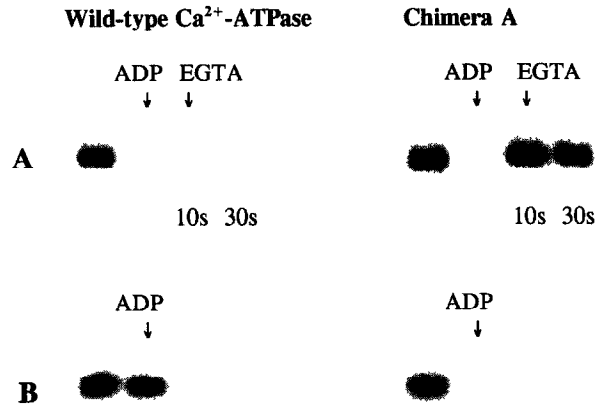

Fig. 6. Examination of the phosphoenzyme intermediates formed from ATP in the wild-type Ca^{2+}-ATPase and Chimera A. (A) Phosphorylation was carried out in the presence of 2 μM [γ-^{32}P]ATP for 15 s at 0 °C in 20 mM MOPS buffer (pH 7.0), 80 mM K^+, 5 mM Mg^{2+}, 0.1 mM Ca^{2+} and 2 μM of the calcium ionophore A23187 (to ensure that the Ca^{2+} concentration was the same on both sides of the membrane). The phosphorylated sample was acid quenched directly (first lane), or the ADP-sensitivity was tested by addition of 1 mM ADP with 1 mM EGTA, followed by acid quenching 5 s later (second lane). To examine the rate of dephosphorylation in the forward direction, 1 mM EGTA was added followed by acid quenching 10 s (third lane) or 30 s (fourth lane) later. (B) Phosphorylation was carried out in the presence of 2 μM [γ-^{32}P]ATP, 100 mM TES/Tris (pH 8.35), 10 mM Mg^{2+}, 50 μM Ca^{2+} and 2 μM calcium ionophore A23187 at 0°C for 15 s. In the first lane, the phosphorylated sample was acid quenched directly. In the second lane, the ADP-sensitivity was tested by addition of 1 mM ADP with 1 mM EGTA followed by acid quenching 5 s later.

for Chimera B than for the wild-type Ca^{2+}-ATPase, this could be accounted for by the lower expression level of Chimera B (cf. Fig. 2). Thus, quantitation of the amount of phosphoprotein on the gel indicated that on a molar basis the phosphorylation of Chimera B was as high as that of the wild-type Ca^{2+}-ATPase. Fig. 5 further illustrates that the phosphorylation from P_i was less sensitive to Ca^{2+} in Chimera B relative to the wild type. In the wild-type Ca^{2+}-ATPase, the back-door phosphorylation from P_i is inhibited by the binding of Ca^{2+} with high affinity, due to displacement of the conformational equilibrium in favor of the E1 form reacting exclusively with ATP and not with P_i. In Chimera B, Ca^{2+} inhibited the phosphorylation from P_i as well, but the Ca^{2+} concentration required for this inhibition was higher (0.1–1 mM) than for the wild type (about 10 μM). These data show that the Ca^{2+} site responsible for inhibition of phosphorylation from P_i was functioning in Chimera B, albeit with reduced affinity.

The finding that Chimera A formed a phosphorylated intermediate from ATP, but not from P_i, combined with the inability of the chimera to transport Ca^{2+}, led us to examine the partial reactions following phosphorylation from ATP, i.e. the conversion of the ADP-sensitive phosphoenzyme intermediate (E1P) to the ADP-insensitive phosphoenzyme intermediate (E2P) and the subsequent hydrolysis of E2P with liberation of P_i. In the experiments described in Fig. 6A, the phosphorylation with ATP was performed at pH 7.0 in the presence of K^+. Under these conditions the major steady-state intermediate was the ADP-sensitive E1P form, both in the wild-type Ca^{2+}-ATPase and in Chimera A, as demonstrated by the complete disappearance of the phosphoenzyme upon a 5s-incubation with ADP. In the presence of ADP, dephosphorylation can occur through reversal of the phosphorylation with resulting synthesis of ATP from ADP and the phosphorylated E1P enzyme. When, on the other hand, EGTA was added without ADP, all the phosphoenzyme present at steady state disappeared rapidly in the wild-type Ca^{2+}-ATPase, but in contrast the dephosphorylation was extremely slow in Chimera A. The addition of EGTA terminates the phosphorylation by chelation of Ca^{2+}, permitting observation of the dephosphorylation of E1P that occurs through conversion of E1P to E2P and hydrolysis of the latter intermediate. The data in Fig. 6A therefore suggested that the E1P→E2P interconversion was blocked in the chimera. This hypothesis was further substantiated by phosphorylation experiments conducted at pH 8.35 in the absence of K^+, as shown in Fig. 6B. In the wild-type Ca^{2+}-ATPase, these conditions favor accumulation of ADP-insensitive E2P in steady state, due to a reduced rate of E2P hydrolysis. Therefore, most of the wild-type Ca^{2+}-ATPase phosphoenzyme (E2P) is seen to remain after the 5s-incubation with ADP. In contrast to the wild type, Chimera A did not accumulate E2P after 15s reaction with ATP in this medium, as demonstrated

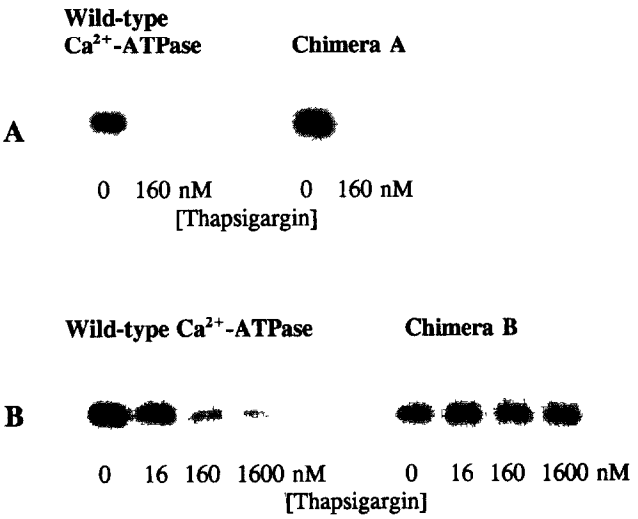

Fig. 7. Effect of thapsigargin on phosphorylation of Chimera A from ATP and of Chimera B from P_i. (A) Microsomal fractions isolated from COS-1 cells transfected with cDNA encoding either wild-type Ca^{2+}-ATPase or Chimera A were incubated for 30 min with or without 160 nM thapsigargin followed by assay of phosphorylation from ATP in the presence of 2 μM [γ-^{32}P]ATP and 100 μM Ca^{2+} as described for Fig. 4. (B) Microsomal fractions isolated from COS-1 cells transfected with cDNA encoding either wild-type Ca^{2+}-ATPase or Chimera B were incubated for 30 min at 25°C with various concentrations of thapsigargin as indicated, followed by assay of phosphorylation from P_i in the presence of 2 mM EGTA as described for Fig. 5.

by the complete dephosphorylation obtained upon addition of ADP. This supports the notion that the conversion of E1P to E2P was blocked in Chimera A.

Thapsigargin, a plant-derived sesquiterpene lactone, has been shown to inhibit SR Ca^{2+}-ATPase activity, but has no effect on the Na^+,K^+-ATPase or on the plasma membrane Ca^{2+}-ATPase [6]. To identify domains in SR Ca^{2+}-ATPase important for the interaction with thapsigargin, we tested the ability of thapsigargin to prevent phosphoenzyme formation in Chimera A and Chimera B. It is seen in Fig. 7A that incubation with a slight molar excess of thapsigargin (160 nM, compare with a concentration of around 50 nM of phosphorylation sites) led to complete inhibition of phosphoenzyme formation from ATP in Chimera A as well as in the wild-type Ca^{2+}-ATPase expressed in COS cells.

In the wild-type Ca^{2+}-ATPase, a molar excess of thapsigargin reduced the phosphorylation level obtained with P_i, as well, to less than 10% (Fig. 7B) consistent with previous observations on purified enzyme from rabbit muscle [5,24]. On the other hand, as seen in Fig. 7B, there was no effect of thapsigargin on P_i phosphorylation in Chimera B. Not even the presence of 1,600 nM thapsigargin, i.e. more than 20-fold molar excess, inhibited the phosphorylation of Chimera B.

4. DISCUSSION

The design of the two chimeric proteins constructed and characterized in the present study was based on a model for P-type ATPases that places ATP-interaction with the cytoplasmic part and ion-binding with the transmembrane part [2–4]. This hypothesis was confirmed by the present data. By constructing chimeric proteins we have demonstrated that parts from two P-type ATPases with different cation selectivity, the SR Ca^{2+}-ATPase and Na^+,K^+-ATPase, are indeed able to supplement each other to some extent, and the cation selectivity seems not to be associated with the central cytoplasmic domain. First of all, it was possible to express the chimeric ATPases in COS-1 cells with proper membrane insertion, since they could be isolated in the endoplasmic reticulum membrane fraction. Neither of the two chimeric ATPases was able to transport Ca^{2+}, but several partial reactions in the enzymatic cycle were found to be intact. CrATP-supported Ca^{2+} occlusion took place in Chimera A, and Ca^{2+} was able to activate phosphorylation from ATP with apparent affinities for Ca^{2+} and ATP very similar to the affinities of wild-type Ca^{2+}-ATPase. The central cytoplasmic domain exchanged in Chimera A contains a number of short segments which are highly conserved within the P-type family, and which based on affinity labeling are believed to interact with ATP. There are, however, also a large number of non-conserved residues, and several gaps in the alignment (Fig. 8), the overall sequence homology between the Ca^{2+}- and Na^+,K^+-ATPase being no more

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349-CSDKTGTLTTNQMSVCKMFIIDKVDGDFCSLNEFSITGSTYAPEGEVLKNDKPIRS
369-CSDKTGTLTQNRMTVAHMWFDN                    QIHEADTTENQSGVSFD

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LSKVERANACNSVIRQLMKKEFTLEFSRDRKSMSVYCSPAKSSRAAVGNKMFVKGAPEGV
KYTKIV                  EIPFNSTNKYQLSIHKNPNASEPK  HLLVMKGAPERI

IDRCNYVRV GTTRVPMTGPVKEKILSVIKEWGTGRDTLRCLALATRDTPPKREEMVLDD
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GIFGENEEVAD                    RAYTGREFDDLPLAEQREACRRA  CCFA
GIISEGNETVEDIAARLNIPVNQVNPRDAKACVVHGSDLKDMTSEELDDILRYHTEIVFA

RVEPSHKSKIVEYLQSYDEITAMTGDGV-705
RTSPQQKLIIVEGCQRQGAIVAVTGDGV-714
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Fig. 8. Alignment of the amino acid sequences of the SR Ca^{2+}-ATPase (upper) and the Na^+,K^+-ATPase (lower) corresponding to the central cytoplasmic domain, which was exchanged in Chimera A. One and two dots indicate conservative replacements and identical residues, respectively.

than 35% in this domain. From the present data it can be concluded that these non-conserved residues in the central cytoplasmic domain are without major importance for determination of cation selectivity and affinity. Moreover, the non-conserved residues can be exchanged without any apparent change in the interaction of the enzyme with ATP.

By contrast, Ca^{2+} did not activate phosphoenzyme formation from ATP in Chimera B, although Ca^{2+} inhibited the phosphorylation from P_i. This finding is most easily interpreted in terms of a stepwise mechanism for binding of two calcium ions, in which the binding of the first calcium ion is sufficient to prevent phosphorylation from P_i, whereas the binding of both calcium ions is required for activation of phosphorylation from ATP [13,22,25]. Based on this model, it seems that the site binding the first calcium ion in the sequence was functioning in Chimera B, whereas the site binding the second calcium ion may have been disrupted. Thus, the data point to a location of the second site in the NH_2-terminal one third of the molecule, which differs between Chimera A and Chimera B. Moreover, since the apparent affinity with which Ca^{2+} inhibited phosphorylation from P_i was somewhat reduced in Chimera B, it is possible that in addition the NH_2-terminal one third of the molecule contributes residues to the first site. The combined evidence from the present data and studies of point mutants [3,7,8,11,13,14] would suggest that the major part of the residues forming the first site are contributed from the putative transmembrane segments M5, M6, and M8, and that M4 (perhaps also M3) constitutes a central element in formation of the second site with some contribution to the first site. A close proximity of the two Ca^{2+} sites is also supported by

kinetic evidence of dissociation of both ions through a common access channel [21,22].

The inability of Chimera A to transport Ca^{2+} could be traced to a block of the E1P→E2P conformational change, the crucial energy transducing event in the transport cycle, which leads to deocclusion of Ca^{2+} at the luminal surface and to loss of ADP sensitivity of the phosphoenzyme [26,27]. In Chimera A, formation of E2P was also blocked in the reverse direction from E2, as indicated by the lack of phosphorylation from P_i. Since the latter function was intact in Chimera B, it seems reasonable to ascribe the lack of E2P formation in Chimera A to a defective interaction of the NH_2-terminal one third of the molecule contributed from Ca^{2+}-ATPase with the central cytoplasmic domain contributed from Na^+,K^+-ATPase. This information supplements previous demonstrations of the involvement of single highly conserved residues in E1P→E2P transition [12].

Finally, the present study provided information on the location of residues of importance for the specific interaction of the SR Ca^{2+}-ATPase with thapsigargin. Thapsigargin has been shown to inhibit high-affinity Ca^{2+} binding, but it is not well understood whether this inhibition is due to a direct interaction with one or both Ca^{2+} binding sites, or thapsigargin acts indirectly by stabilizing a conformation (E2) with low affinity [5,24]. Since Chimera A was inhibited by thapsigargin, whereas Chimera B was not, it appears that residues conferring thapsigargin sensitivity are located in the NH_2-terminal one third of the molecule. Recently, it was reported that the first 200 amino acid residues of the Ca^{2+}-ATPase are without importance for thapsigargin sensitivity [28]. In combination with our result this data places the residues crucial to thapsigargin binding within the segment between residue 200 and residue 348. On the basis of the hydrophobic nature of thapsigargin one would suggest that transmembrane segments M3 and/or M4 might be involved.

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