Mutational analysis of trans-membrane helices M3, M4, M5 and M7 of the fast-twitch Ca^{2+} -ATPase

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Mutational analysis of trans-membrane helices M3, M4, M5 and M7 of the Ca^{2+} -ATPase revealed a novel phenotypic variant, M4 [Y295A (the one-letter symbols are used for amino acid residues throughout)], displaying an increased affinity for P_i and decreased affinity for MgATP, while retaining the ability to translocate Ca^{2+} ions across the endoplasmic reticulum membrane. The properties of this mutant suggest that the E1–E2 equilibrium is shifted towards E2, and indicate a key role for this aromatic residue (Y295) at the end of trans-membrane helix M4. A mutant containing three amino acid residue substitutions at the end of the seventh trans-membrane helix, M7 (F834A, F835A, T837F),

showed a complete loss of ATPase activity and a reduced ability to phosphorylate with P_i , although MgATP-initiated phosphorylation was unaffected. The observation that single mutations in this cluster of residues had no effect on Ca^{2+} transport suggests that correct anchoring of the helix at the lipid–water interface by these aromatic residues is important in the functioning of the ATPase. Mutation of polar residues in helix M3 did not affect inhibition of the ATPase by thapsigargin, thapsivillosin A or t-butyl hydroquinone, suggesting that hydrogen-bonding partners for the essential -OH groups on these inhibitors lie elsewhere in the ATPase.

INTRODUCTION

The Ca^{2+} -ATPase [sarco/endoplasmic reticulum Ca^{2+} -ATPase isoform 1a (SERCA 1a)] of skeletal muscle sarcoplasmic reticulum (SR) is a member of the P-type ion-translocating ATPases, a family of proteins that includes the plasma membrane Ca^{2+} -ATPase [1], the Na^+/K^+ -ATPase [2], the H^+ -ATPase [3] and the H^+/K^+ -ATPase [4], which form a phosphorylated intermediate during their catalytic cycle. The protein catalyses the translocation of 2 Ca^{2+} ions across the SR membrane for each molecule of ATP hydrolysed. Binding of Ca^{2+} to the ATPase from the cytoplasmic side of the SR membrane is co-operative and relatively slow, involving a conformational change on the ATPase [5–8]. After phosphorylation of the ATPase by ATP, the high-affinity, cytoplasmically exposed sites are no longer able to bind Ca^{2+} , the Ca^{2+} ions being transferred to a pair of low-affinity sites on the luminal side of the membrane, from where the Ca^{2+} ions can be released into the lumen of the SR (Scheme 1). The scheme, as demonstrated by Myung and Jencks (see [9]), includes only one Ca^{2+} -bound phosphorylated form of the ATPase, E1PCa_2 . This contrasts with the Ca^{2+} -bound phosphorylated forms E1PCa_2 and E2PCa_2 in the original scheme presented by de Meis and Vianna [10]. In terms of Scheme 1,

Scheme 1

ADP-sensitive ATPase is E2PCa_2 and ADP-insensitive ATPase is E2P .

The Ca^{2+} -ATPase is predicted to contain ten trans-membrane α -helices [11], which is consistent with a variety of labelling and antibody-binding experiments [12,13] and with low resolution electron microscopy data [14]. The site-directed mutagenesis experiments of Clarke et al. [11,15] showed that the high-affinity Ca^{2+} -binding sites are located in the trans-membrane region of the ATPase, between putative trans-membrane α -helices M4, M5, M6 and M8, each of which contains one negatively charged residue. It has been proposed that helices M4, M5, M6 and M8 are packed in a square array, making a channel-like structure in which the two Ca^{2+} ions bind with high affinity, one above the other [14,16]. Phosphorylation of the ATPase, with transfer of the two bound Ca^{2+} ions from the pair of high-affinity sites to a second pair of low-affinity sites, presumably involves a change in the packing of these four helices. It has also been suggested that the packing of the four helices changes during the Ca^{2+} binding process, the initial E1 conformation of the ATPase only possessing a single Ca^{2+} ion binding site, with binding of the first Ca^{2+} ion leading to a change in the packing of the helices and the creation of the second Ca^{2+} ion binding site [16,17].

Correct packing of the helices M4, M5, M6 and M8 depends, at least in part, on having the correct thickness for the surrounding lipid bilayer. If the Ca^{2+} -ATPase is reconstituted into phosphatidylcholine bilayers which are too thin (with a phospholipid fatty acyl chain length of C_{14}) or too thick (with a phospholipid fatty acyl chain length of C_{24}), the Ca^{2+} binding stoichiometry changes from the usual 2 Ca^{2+} ions bound per ATPase molecule to 1 Ca^{2+} ion bound per ATPase molecule, showing that helix packing must have changed [18]. It has been suggested that correct insertion of α -helices into lipid bilayers is favoured by the presence of aromatic residues at the ends of trans-membrane α -helices, anchoring the helix firmly into the

Abbreviations used: BHQ, 2,5-di(t-butyl)-1,4-benzohydroquinone; SERCA1a, sarco/endoplasmic reticulum Ca^{2+} -ATPase isoform 1a; SR, sarcoplasmic reticulum; TG, thapsigargin; TvA, thapsivillosin A; UTR, untranslated region.

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lipid bilayer [19]. Alignments of trans-membrane helices for the Ca^{2+} -ATPase family suggest that clusters of aromatic residues may be located at the ends of two helices in the Ca^{2+} -ATPase, M4 and M7 [20]. In this paper we report on the effects of mutating these residues.

Helix-helix interactions will also be important in determining helix packing in the Ca^{2+} -ATPase. It has been established that specific interactions occur between trans-membrane α -helices of membrane proteins so that, for example, a native polytopic membrane protein can be formed by specific aggregation of separately expressed fragments of the protein [21]. Interactions between helices M4, M5, M6 and M8 and the other α -helices in the ATPase are, therefore, likely to be important features of the ATPase structure. Of these other helices, specific functions have been assigned to helices M3 and M7. Helix M7, predicted to be longer than the other helices, has been suggested to be inclined relative to the other helices; the unusual orientation of helix M7, combined with the fact that it lies between two helices (M6 and M8) involved in Ca^{2+} binding, make helix M7 an attractive candidate for linking phosphorylation of the ATPase to changes in the packing of the helices [14]. An important role for helix M3 is suggested from studies of the binding of the potent and specific inhibitor of the Ca^{2+} -ATPase, thapsigargin (TG), a sesquiterpene lactone. Binding of TG to the Ca^{2+} -ATPase results in a decreased affinity of the ATPase for Ca^{2+} [22], resulting from a conformational change on the ATPase [23]. The marked hydrophobicity of TG makes it likely that its binding site involves the trans-membrane region of the ATPase. Studies with chimaeras between the Ca^{2+} -ATPase and the Na^+/K^+ -ATPase have suggested that the binding site for TG could involve helix M3 [24–27], which is also consistent with photo-labelling experiments [28]. Helix M3 is unusual in containing a large number of polar residues which could be important in inhibitor binding, forming hydrogen-bonding interactions with the two -OH groups in the sesquiterpene lactone inhibitors which have been shown to be essential for activity [29]. The presence of two -OH groups is also important for inhibition of the Ca^{2+} -ATPase by other hydrophobic molecules, such as 2,5-di(*t*-butyl)-1,4-benzohydroquinone (BHQ), which inhibit by a mechanism similar to TG, but bind to a different site on the ATPase [30]. We have also therefore mutated the polar residues in helix M3, and the unusual pair of Ser residues in helix M5, to define their roles in inhibitor binding and ATPase function.

MATERIALS AND METHODS

Construction of a semi-synthetic gene coding for the Ca^{2+} -ATPase

Plasmid pATH10-FCA1 (a gift from Professor D. H. MacLennan, University of Toronto, Canada), which contains a truncated cDNA coding for residues 32 to 949 of the rabbit fast-twitch Ca^{2+} -ATPase (SERCA 1), was employed as the starting material for the construction of a full-length gene. The 5' and 3' termini of this gene were constructed using synthetic DNA fragments. A DNA fragment containing a modified 5' untranslated leader region (UTR) with useful restriction enzyme sites and also coding for the N-terminal 32 amino acid residues, was inserted into pATH10-FCA1 as an *EcoRI*–*XbaI* fragment. Subsequently, a second fragment, containing further restriction-enzyme sites in a modified 3' UTR and coding for the final 52 residues, was inserted at the 3' end using *SalI* and *ClaI* restriction sites. The entire gene was sequenced to confirm the primary structure and the full-length cDNA was subcloned into the vector pcDNA1 (Invitrogen) for expression. The synthetic cDNA codes for the rabbit fast-twitch SERCA1 protein sequence as given in the Swiss-Prot database (accession number P04191).

M3	M4	M5	M7	
L	T	M	G	CYTOSOL
S261A	T	K	W	
K	I	Q	L	
V	V	F	F834A	MEMBRANE
I	A	I	F835A	
S265A	P	R	R	
L	L	Y763F	Y837F	
I	G	L	M	
C268A	E	I	A	
V	P	S766A	I	
A	I	S767A	G	
V	A	N	G	
W272A	A	V	Y	
L	V	G	V	
I	A	E	G	
N	L	V	A	
I	A	V	A	
G	V	C	T	
H278A	A	I	V	
F	I	F	G	
N	K	L	A	
D281A	F	T	A	
P	Y295A	A	WWFM	
V	Y294A	A	Y	LUMEN
H	I	L	A	
G	A	G	E	

Figure 1 Predicted location of the mutations examined in this study

Bold typeface indicates the altered residues contained in the multiple mutants described in Table 1. The estimated position of the membrane is shown.

Site-directed mutagenesis of the ATPase cDNA was carried out using the oligonucleotide-directed *in vitro* mutagenesis method described by Kunkel et al. [31] or using the QuikChange site-directed mutagenesis kit (Stratagene). The mutations described in this study are shown in Figure 1.

Expression of the Ca^{2+} -ATPase in COS-1 cells

Monolayers of COS-1 cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL) containing 10% (v/v) fetal calf serum, 4 mM L-glutamine, 50 µg/ml gentamicin and 2.5 µg/ml amphotericin B under 5% CO_2 /95% air at 37 °C. DNA transfection was carried out using the lipofection reagent Lipofectamine™ (Gibco-BRL) and following the instructions supplied. Transfected cell cultures were incubated in complete medium for 3 days before harvesting and preparation of microsomal fractions (see below).

Preparation of a microsomal fraction and immunodetection of expressed ATPase

Microsomes from cultured COS-1 cells were prepared using a similar method to that described by Maruyama and MacLennan [32]. Cells from a 175 ml tissue-culture flask were washed twice in 10 ml of PBS and once in PBS containing 5 mM EDTA. The cells were swollen at 0 °C for 10 min in 10 ml of a hypotonic solution of 10 mM Tris/HCl/0.5 mM MgCl_2 , pH 7.5; PMSF was then added to a final concentration of 0.2 mM. The cells were homogenized with 30 strokes in a glass Dounce homogenizer and the homogenate was diluted with an equal volume of 0.5 M sucrose/6 mM 2-mercaptoethanol/40 µM CaCl_2 /300 mM KCl/20 mM Tris/HCl, pH 7.5, and homogenized again. The suspension was centrifuged at 10000 *g* for 20 min to pellet cell debris, and the supernatant was made 0.6 M in KCl by the addition of 0.9 ml of a 2.5 M solution. The suspension was centrifuged at 100000 *g* for 60 min and the microsomal pellet was resuspended in the same buffer and centrifuged again. The

final pellet was resuspended in a solution of 0.25 M sucrose/3 mM 2-mercaptoethanol/20 μM CaCl_2 /150 mM KCl/10 mM Tris/HCl, pH 7.5, at a protein concentration of approx. 1 mg/ml.

The expressed ATPase was visualized by immunoblotting of microsome preparations from transfected cells. Immunoreactive protein was detected using the mouse monoclonal antibody 1/2H7, raised against the phosphorylation domain of rabbit Ca^{2+} -ATPase [33], and a horseradish peroxidase-conjugated secondary antibody raised against mouse antibodies (sheep anti-mouse IgG; Sera-labs). Expression levels were quantified by densitometric scanning of immunoreactive bands derived from transfected COS-1 cells and authentic fast-twitch ATPase.

Calcium transport assay

Active transport of calcium into microsomes prepared from cultured cells was assayed using the procedure described by Maruyama and MacLennan [32]. Uptake was initiated by the addition of purified microsomes, containing 1–1.5 μg of recombinant protein, into the transport buffer: 20 mM Mops/KOH, pH 7.0, 100 mM KCl, 5 mM MgCl_2 , 5 mM ATP, 100 μM free Ca^{2+} (containing $^{45}\text{Ca}^{2+}$ at a specific activity of 10^6 c.p.m./ μmol) and 5 mM potassium oxalate. Oxalate was included in the transport buffer (as described in [11,13,15,27,32,34]) to permit efficient loading of vesicles by functionally active recombinant ATPases and prevent Ca^{2+} efflux across vesicle membranes. At various time points, 100 μl samples were filtered through a 0.22 μm Millipore filter and washed with 10 ml of 0.1 M KCl. Radioactivity on the filters was measured by liquid-scintillation counting.

Inhibitor studies

To determine the sensitivity of the mutant forms of the protein to the specific inhibitors TG (Sigma), thapsivillosin A (TvA; extracted from *Thapsia vellosa* roots as described in Wictome et al. [23]) and BHQ (Aldrich) active uptake of Ca^{2+} into microsomes prepared from cells expressing the different proteins was measured in the presence and absence of various concentrations of each inhibitor. TG and TvA (nM final concentrations) and BHQ (μM final concentrations) were added to the reactions as 1000 $\times$ concentrated stock solutions in DMSO.

Phosphoenzyme formation

Phosphoenzyme formation was determined by mixing microsomes with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in ice-cold E1 or E2 buffer (20 mM Mops, pH 7.4/5 mM MgCl_2 /100 mM KCl, and either 2 mM EGTA or 100 μM free Ca^{2+} respectively) as described [15]. Briefly, 50 μl of ice-cold E1 or E2 buffer was added to microsomal preparations containing 0.5–1 μg of recombinant ATPase. The reaction was started at room temperature by the addition of 10 μl of 0.5 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (10^6 c.p.m./nmol) and stopped 10 s later by the addition of 1 ml of 0.6 M perchloric acid and 1 mM phosphoric acid. After centrifugation for 15 min at 14000 g, the protein pellets were resuspended in 20 μl of final sample buffer [1% (w/v) SDS/6 M urea/1% (v/v) 2-mercaptoethanol/2 mM EDTA/50 mM Tris/HCl, pH 6.5]. Incorporation of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was detected by acidic SDS/PAGE [35] and autoradiography.

Phosphorylation with P_i was carried out in 100 μM $^{32}\text{P}_i$ (10 μCi)/100 mM Mops/KOH (pH 7.0)/5 mM MgCl_2 /20% (v/v) DMSO in the presence or absence of 100 μM free Ca^{2+} for 10 min at 25 $^{\circ}\text{C}$, as outlined by Andersen et al. [34]. The radioactive samples were precipitated with a mixture of 0.6 M perchloric acid and 1 mM phosphoric acid and separated by acidic SDS/PAGE as described above.

Measurement of ATP hydrolysis

The rate of ATP hydrolysis was measured spectrophotometrically using an NADH-coupled assay as described by Vilsen et al. [36]. Turnover rates were measured at 37 $^{\circ}\text{C}$ in a buffer containing 0.15 mM NADH/1 mM phosphoenolpyruvate/10 units/ml lactate dehydrogenase/10 units/ml pyruvate kinase/20 mM Mops/KOH (pH 7.2)/100 mM KCl/5 mM MgCl_2 /2 mM ATP/1 mM EGTA/1 μM calcium ionophore A23187, in the presence or absence of 50 μM free Ca^{2+} .

RESULTS AND DISCUSSION

Construction of a semi-synthetic gene coding for SERCA1

To facilitate subsequent manipulations and mutagenesis we constructed a semi-synthetic gene specifying the SERCA1 isoform of the Ca^{2+} -ATPase. Using unique restriction enzyme sites engineered into the 5' and 3' flanking sequences, the gene was subcloned into the expression vector pcDNA1 and the final construct was introduced into monolayers of COS-1 cells by a lipofectamine-mediated transfection procedure. Expression of the recombinant Ca^{2+} -ATPase was shown by immunoblotting using a monoclonal antibody specific for the native Ca^{2+} -ATPase (Figure 2). The protein had a molecular mass that was indistinguishable from the wild-type enzyme and was shown to localize efficiently in the endoplasmic reticulum of COS-1 cells after transfection (Figure 2). In addition, the recombinant ATPase was capable of translocating Ca^{2+} ions in an ATP-

Figure 2 Immunoblot analysis of microsome preparations from COS-1 cells transfected with the wild-type Ca^{2+} -ATPase DNA or mutant forms of the gene

The proteins were separated by SDS/PAGE, transferred onto nitrocellulose membranes and the ATPase was detected by incubation with the monoclonal antibody 1/2H7 (directed against the phosphorylation domain of the Ca^{2+} -ATPase) as described in the Materials and methods section. Equal amounts of each microsomal fraction were loaded onto the gels, and the signal intensity for each mutant was compared with that of a selected wild-type recombinant ATPase sample. (a) Mutant proteins containing single amino acid residue substitutions. Lanes: +, wild-type recombinant protein; -, microsomes prepared from cells transfected with the parent expression vector pcDNA1; 1, S261A; 2, S265A; 3, C268A; 4, H278A; 5, D281A; 6, F835A; 7, F835A; 8, Y837F; 9, Y294A; 10, Y295A. (b) Mutant proteins containing multiple amino acid residue substitutions. Lanes: +, wild-type recombinant protein; -, microsomes prepared from cells transfected with the parent expression vector pcDNA1; 1, M3 (S265A, W272A); 2, M4 (Y294A, Y295A); 3, M5 (Y763F, S766A, S767A); 4, M7 (F834A, F835A, Y837F). The positions of molecular mass markers (in kDa) are shown on the left.

Table 1 Expression levels of recombinant wild-type and mutant forms of the Ca^{2+} -ATPase in COS-1 cells and their ability to translocate Ca^{2+}

Expression levels of the mutant proteins were determined by comparison with the recombinant wild-type ATPase after Western blot analysis of microsomal preparations and densitometry of the immunoreactive bands. Levels of calcium accumulated by the mutant ATPases over a time period of 30 min were determined and expressed as a percentage of the level of accumulation (520 nmol Ca^{2+} /mg of total protein, equivalent to 22 μmol Ca^{2+} /mg of ATPase over 30 min) by wild-type recombinant ATPase; Ca^{2+} accumulation by untransfected cells was 45 nmol Ca^{2+} /mg of total protein (equivalent to 2 μmol Ca^{2+} /mg of ATPase) over 30 min. This basal level of Ca^{2+} accumulation was subtracted from the maximum levels of Ca^{2+} transport achieved by the recombinant wild-type and mutant ATPases, and these values are expressed as a percentage of the maximal Ca^{2+} accumulation achieved by the wild-type enzyme.

Mutant	Location	Expression level relative to wild-type (%)	Maximal Ca^{2+} accumulation relative to wild-type (%)
S261A	M3	110	110
S265A	M3	101	107
C268A	M3	108	109
H278A	M3	93	110
D281A	M3	89	101
Y294A	M4	91	97
Y295A	M4	99	29
F834A	M7	100	92
F835A	M7	107	101
Y837F	M7	99	103
M3 (S265A, W272A)	M3	108	106
M4 (Y294A, Y295A)	M4	94	30
M5 (Y763F, S766A, S767A)	M5	98	0
M7 (F834A, F835A, Y837F)	M7	48	0

dependent manner at similar rates to the native protein (see below).

Production and expression of mutants altered in trans-membrane helices M3, M4, M5 and M7

Mutations introduced into helices M3, M4, M5 and M7 of the ATPase are listed in Table 1 (the one-letter symbols are used for amino acid residues throughout). Most were single mutations, although three recombinant proteins containing multiple substitutions in M4 (Y294A, Y295A), M5 (Y763F, S766A, S767A) or M7 (F834A, F835A, Y837F) were also produced. The predicted locations of these residues are shown in Figure 1.

Immunoblot analyses of microsomes from cells expressing the wild-type or mutant calcium pumps indicated that all were correctly targeted to the membrane fraction (Figure 2). Comparison of the levels of wild-type and mutant forms of the protein with authentic native rabbit Ca^{2+} -ATPase indicated that the recombinant form of the wild-type protein was present at a concentration of approx. 25 μg /mg of total protein, indicating that the ATPase constitutes approx. 2.5% of the microsomal fraction. This is in good agreement with the measurements made by Sumbilla et al. [24] using a similar expression system. Table 1 shows the relative expression levels of the wild-type ATPase and of all of the mutant forms of the protein examined in this study.

Calcium transport studies

Single mutations of the polar residues in helix M3 had no effect on the level of Ca^{2+} accumulated by the Ca^{2+} -ATPase; the double mutation S265A, W272A was also without effect on Ca^{2+} transport (Table 1). Similarly, single mutations of the three

Figure 3 ATP-induced phosphorylation of the wild-type Ca^{2+} -ATPase and mutants with altered calcium transport function

Phosphorylation with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was carried out in the presence (+) or absence (–) of 100 μM free Ca^{2+} . Track 1, pcDNA1 (parent expression vector lacking ATPase gene); track 2, wild-type (WT); track 3, Y295A; track 4, M5 (Y763F, S766A, S767A); track 5, M7 (F834A, F835A, Y837F). Samples containing an equivalent amount of expressed ATPase (for the pcDNA1 analysis, an amount of total protein equivalent to that used in the wild-type experiment was used) were separated by PAGE performed at pH 6.4, and bands were detected and quantified using a phosphorimager. The positions of molecular mass markers (in kDa) are shown on the left.

aromatic residues predicted to lie at the end of helix M7 (F834, F935, and Y837) did not affect Ca^{2+} transport. However, simultaneous mutation of all three aromatic residues totally abolished Ca^{2+} transport (Table 1). Of the two aromatic residues predicted to lie at the end of helix M4, mutation of Y294 had no effect, whereas mutation of Y295 reduced the level of Ca^{2+} uptake to 30% of the wild-type value; the level of Ca^{2+} accumulation measured for the double mutant (Y295A, Y294A) was the same as that for the single mutation Y295A (Table 1).

Clarke et al. [37] and Rice and MacLennan [38] have reported that single mutations S766A and S767A in helix M5 reduced the Ca^{2+} affinity of the ATPase, and suggested that these two residues could be part of the lining of a polar channel allowing access of Ca^{2+} to its binding sites in the pump. The mutation Y763G has been reported to uncouple Ca^{2+} transport from ATP hydrolysis [39], and the mutations Y763T [38] and Y763A [40] also reduced Ca^{2+} uptake, but the mutation Y763F had no functional consequences [38]. On the basis of these experiments it was suggested that Y763 could act as a bulky cap to the pore, and that uncoupling in mutant Y763G could be due to unique properties of the Gly residue [38]. We have found that the triple mutant Y763F, S766A, S767A showed no accumulation of Ca^{2+} (Table 1).

The mechanism of Ca^{2+} transport deficiency

The mechanism of the Ca^{2+} -ATPase is usually described in terms of the E1–E2 model (Scheme 1) [10]. The scheme proposes that, in the E1 conformation, the ATPase has two outward-facing Ca^{2+} -binding sites of high affinity. After the binding of MgATP, the ATPase is phosphorylated and undergoes a change in conformation to a state in which the two Ca^{2+} -binding sites are of low affinity and inward facing (E2 PCa_2). After loss of Ca^{2+} to the lumen of the SR, the ATPase can dephosphorylate and recycle to E1. Binding of Ca^{2+} to high-affinity exterior-facing sites on the ATPase causes a change in chemical reactivity for the ATPase, from being reactive to P_i and H_2O in the E2 state to

Figure 4 Phosphorylation and dephosphorylation of mutants Y295A and M7(F834A, F835A, Y837F)

Phosphorylation was initiated in the presence of Ca²⁺ for 20 s as described in the Materials and methods section. Dephosphorylation was initiated by the addition of 1 mM ADP and 1 mM EGTA 5 s before quenching. Track 1, wild-type; track 2, Y295A; track 3, M7 (F834A, F835A, Y837F): (—) no addition of ADP and EGTA, (+) addition of ADP and EGTA. Samples containing an equivalent amount of ATPase were separated by electrophoresis at pH 6.4. The positions of molecular mass markers (in kDa) are shown on the left.

being reactive with ATP and ADP in the E1 state. A study of partial reactions of the ATPase can define the mechanism of the loss of Ca²⁺ transport by the mutants.

As shown in Figure 3, mutant M4 (Y295A) and the triple mutant M7 (F834A, F835A, Y837F) were phosphorylated by [γ -³²P]ATP in a Ca²⁺-dependent manner. However, densitometry of the phosphorylated bands showed that whereas the mutant M7 (F834A, F835A, Y837F) was phosphorylated to the same extent as the wild-type protein, the mutant M4 (Y295A) was only phosphorylated to about 60 % of the level of the native ATPase. Addition of ADP to the phosphorylated proteins led to loss of phosphoprotein, showing that the phosphoproteins were ADP-sensitive (Figure 4). In contrast to these results, mutant M5 (Y763F, S766A, S767A) was not phosphorylated by ATP (Figure 3), suggesting changes either at the Ca²⁺-binding sites on the ATPase or at the nucleotide binding or phosphorylation domains.

Effects of mutations on the Ca²⁺-binding sites can be detected through effects of Ca²⁺ on phosphorylation of the ATPase by P_i which, normally, can only occur in the absence of Ca²⁺. In the presence of 20 % DMSO and the absence of Ca²⁺ the wild type ATPase, the mutant M4 (Y295A) and the triple mutant M5 (Y763F, S766A, S767A) were phosphorylated to the same extent (Figure 5). The triple mutant M7 (F834A, F835A, Y837F) was also phosphorylated by P_i, but to a level of about 20 % of that of the wild-type protein. Phosphorylation of the mutant M4 (Y295A) and the triple mutant M7 (F834A, F835A, Y837F) was inhibited by the presence of Ca²⁺, as for the wild-type ATPase (Figure 5). However, the presence of 100 μ M Ca²⁺ had no effect on phosphorylation of the triple mutant M5 (Y763F, S766A, S767A) by P_i, suggesting that this mutant was unable to bind Ca²⁺ to the high-affinity cytoplasmic sites; this observation explains the lack of Ca²⁺-dependent phosphorylation by ATP shown in Figure 3.

The mutant M4 (Y295A) was fully phosphorylated by P_i in the presence of just 10 % DMSO, conditions under which the level of phosphorylation of the native ATPase was reduced (Figure 5b). This increased level of phosphorylation by P_i, combined with the lower level of phosphorylation observed with ATP (Figure 3),

Figure 5 P_i-induced phosphorylation of wild-type (WT) and mutant ATPases

(a) Phosphorylation of the WT ATPase and mutants Y295A, M5 (Y763F, S766A, S767A) and M7 (F834A, F835A, Y837F) with [γ -³²P]P_i in the presence of 20 % DMSO, and in the presence (+) or absence (—) of 100 μ M free Ca²⁺. Track 1, wild-type; track 2, Y295A; track 3, M5 (Y763F, S766A, S767A); track 4, M7 (F834A, F835A, Y837F). Samples containing an equivalent amount of ATPase were separated by electrophoresis at pH 6.4. (b) Phosphorylation of the wild-type ATPase and mutants Y295A, M5 (Y763F, S766A, S767A) and M7 (F834A, F835A, Y837F) with [γ -³²P]P_i in the absence of Ca²⁺ and in the presence of 10 % DMSO. Lane 1, wild-type; lane 2, Y295A; lane 3, M5 (Y763F, S766A, S767A); lane 4, M7 (F834A, F835A, Y837F). This experiment was repeated twice with different microsomes preparations containing the wild-type and mutant proteins. The positions of molecular mass markers (in kDa) are shown on the left.

suggests that the mutation Y295A could stabilize the E2 form of the ATPase, relative to the E1 form.

The observation that the triple mutant M7 (F834A, F835A, Y837F) was phosphorylated by both ATP and by P_i, but did not transport Ca²⁺, suggests either that the mutant was blocked in a step following phosphorylation or that ATP hydrolysis was uncoupled from Ca²⁺ accumulation. To distinguish between these alternatives we measured the ATPase activity of the mutant, using the NADH-coupled assay system described by Vilsen et al. [36]. As shown in Figure 6, Ca²⁺-dependent ATPase activity was readily measured for the wild-type ATPase at a rate of 2.82 μ mol/min per mg of ATPase. However, ATPase activity was not detected in microsomes containing the triple mutant M7 (F834A, F835A, Y837F). We

Figure 6 Ca^{2+} -dependent ATPase activity of the wild-type and M7 (F834A, F835A, Y837F) mutant proteins

ATPase activity was measured spectrophotometrically using an NADH-coupled assay system described in the Materials and methods section. The change in absorbance at 340 nm was measured over time after the addition of microsomes containing (A) the wild-type and (B) M7 mutant (F834A, F835A, Y837F) ATPase. 1, Addition of microsomes; 2, addition of 100 μM free Ca^{2+} ; 3, addition of 5 μM ionophore A23187. The scale bar represents an absorbance of 0.1.

conclude therefore that this mutant is blocked in a step following phosphorylation by ATP. If this block were in the rate of dephosphorylation of E2P, we would expect E2P to build up at steady state in the presence of ATP, and the phosphoenzyme formed would then be insensitive to ADP, since only E2PCa_2 can bind ADP to regenerate ATP (Scheme 1). The observation that the phosphoenzyme formed by the triple mutant was, in fact, ADP-sensitive therefore suggests that the block in activity followed from a decreased rate of Ca^{2+} dissociation from E2PCa_2 .

Residues S261, S265, C268, W272, H278, D281, Y294, Y295, F834, F835 and Y837 are not involved in binding the specific inhibitors TG and BHQ

All of the functionally active proteins described here were tested for sensitivity to the inhibitors TG and BHQ (see Figure 7). These recombinant proteins displayed similar levels of sensitivity to each inhibitor, with K_i values of 3–5 nM and 2–5 μM for TG and BHQ respectively (see Table 2). We also tested the effects of TvA on mutations made in M3 (see Figure 8). TvA is a weaker inhibitor of the Ca^{2+} -ATPase than TG [23] and, as shown in Figure 8, 10 nM TvA only results in approx. 50% inhibition of Ca^{2+} uptake compared with the almost total inhibition observed with 10 nM TG (Figure 7, top). Importantly, inhibition of wild-type Ca^{2+} -ATPase by TvA is indistinguishable from that of the mutants. We therefore conclude that the targeted residues do not appear to be involved in binding either the sesquiterpene lactones or BHQ and so can be eliminated from the search for the inhibitor binding sites.

Figure 7 TG and BHQ sensitivity

Top: sensitivity of the wild-type protein (▲), and mutants S265A (○), C268A (▼), and H278A (●) to TG. Control microsomes (○), prepared from cells transfected with the parent expression vector pcDNA1, are also shown. The levels of calcium accumulated by microsome preparations was measured after 30 min in the presence of TG at the indicated concentrations. The K_i (TG) for each protein was determined from the graphs; a complete list of the K_i values for all the mutants is shown in Table 2. Error bars indicate the range of points about the mean. Bottom: sensitivity of wild-type ATPase (▲), and mutants S265A (○), C268A (▼) and H278A (◆) to the inhibitor BHQ. Control microsomes (●), prepared from cells transfected with the parent expression vector pcDNA1, are also shown. Calcium accumulation of microsomes was measured after 30 min in the presence of various concentrations of inhibitor. The K_i (BHQ) for each protein was determined from the graphs; a complete list of the K_i values for all the mutants is shown in Table 2. Error bars indicate the range of points about the mean.

Conclusions

Helices M4, M5, M6 and M8 make up the two high-affinity Ca^{2+} -binding sites on the Ca^{2+} -ATPase [11,15]. The correct packing of

Table 2 K_i values for TG and BHQ for the recombinant wild-type ATPase and functionally active mutants described in this work

The values were determined from Ca²⁺ transport experiments performed in the presence of different concentrations of each inhibitor (see Figure 7).

Mutant	Location	K_i for TG (nM)	K_i for BHQ (μ M)
WT	—	3–5	0.15–0.2
S261A	M3	2.5	0.2
S265A	M3	4	0.2
M3 (S265A, W272A)	M3	3	0.15
C268A	M3	3.5	0.2
H278A	M3	3.5	0.15
D281A	M3	3.5	0.2
Y294A	M4	4	0.2
Y295A	M4	2.5	0.15
F834A	M7	3	0.2
F835A	M7	3	0.2
Y837F	M7	4	0.2

Figure 8 Sensitivity to TvA

Sensitivity of the wild-type protein (●) and mutants S261A (○), M3 (S265A, W272A) (▲), C268A (▼), H278A (△) and D281A (▽) to TvA. Control microsomes (□), prepared from cells transfected with the parent expression vector pcDNA1, are also shown. The levels of calcium accumulated by microsome preparations was measured after 30 min in the presence of TvA at the indicated concentrations. Error bars indicate the range of points about the mean.

these helices depends on appropriate interaction with other helices in the ATPase and with the surrounding lipid bilayer. Changes in lipid bilayer thickness can change the stoichiometry of Ca²⁺ binding to the ATPase [18]. It has been suggested that aromatic residues located at the ends of helices can serve to anchor a helix correctly into a lipid bilayer [19]. Helix M4 contains a pair of Tyr residues (294, 295) at its luminal end (Figure 2), conserved in the SERCA family [20]. Mutation of Y294 to Ala had little effect on Ca²⁺ accumulation, but mutation of Y295 to Ala markedly reduced Ca²⁺ accumulation (Table 1).

Reduced phosphorylation by ATP (Figure 3) and increased phosphorylation by P_i (Figure 5) would suggest that the mutation could favour the E2 conformation of the ATPase. Helix M7 contains a Tyr residue and a pair of Phe residues at its cytoplasmic end (Figure 1), conserved in the SERCA family, although the two Phe residues are absent from the putative Ca²⁺-ATPases of *Plasmodium falciparum* and *Plasmodium yoelii* [20]. Mutation of any of these residues singly to Ala had no effect on accumulation of Ca²⁺, but simultaneous mutation of all three to Ala led to loss of the ability to accumulate Ca²⁺ (Table 1). The observation that the triple mutant was phosphorylated by both ATP and P_i (Figures 3 and 5), but that the steady-state rate of hydrolysis of ATP was very low (Figure 6) and that the phosphorylated ATPase formed from ATP was ADP-sensitive (Figure 4), suggested that the ATPase was blocked in the E2PCa₂, with slow Ca²⁺ dissociation from the phosphorylated ATPase. This would be consistent with the suggestion [14] that helix M7 is important in linking phosphorylation of the protein to changes in the Ca²⁺-binding sites on the ATPase and that correct anchoring of helix M7 at the lipid–water interface by the aromatic residues is important in the functioning of the ATPase.

In general, conserved polar residues in trans-membrane α -helices are believed to be involved in helix–helix interactions or to provide the linings of trans-membrane pores or ligand-binding sites. Helix M3, believed to contact either helix M5 or helix M4 in the ATPase [17, 41], is unusual in containing a number of polar residues conserved in the Ca²⁺-ATPase family (C268, H278, D281) and a conserved Trp residue at position 272 [20]. Mutation of these residues, however, does not affect accumulation of Ca²⁺ by the ATPase (Table 1). Furthermore, it has been suggested that helix M3 is important in the binding of the inhibitor TG to the ATPase. As shown in Figure 7 and Table 2, mutation of S261, S265, C268, W272, H278 or D281 has no detectable effect on inhibition by TG. Because the binding constant for TG has been estimated to be $4 \times 10^{10} \text{ M}^{-1}$ [23] it is possible that a small decrease in affinity for TG would go undetected in the experiment shown in Figure 7. Therefore, we also studied inhibition by TvA, for which the binding constant for the ATPase is estimated to be $4 \times 10^8 \text{ M}^{-1}$ [23]. As shown in Figure 8, the weaker binding of TvA means that only approx. 50% inhibition is observed at 10 nM TvA. However, the level of inhibition for the wild-type and mutant ATPases is identical within experimental error, showing that these mutations in M3 do not alter the binding constant for the sesquiterpene lactones. It is also shown in Figure 7 that mutation of these residues in M3 does not affect the affinity for BHQ.

It has been shown that the two -OH groups in the sesquiterpene lactones and in BHQ are essential for inhibition [29, 30], suggesting the importance of hydrogen-bonded interactions with suitable residues in the ATPase. The results shown in Figures 7 and 8 suggest that these hydrogen-bonding partners cannot be in helix M3. We have also shown that residues Y294 and Y295 in Helix M4 and residues F834, F835 and Y837 in helix M7 cannot be important in inhibitor binding (Table 2). It therefore seems likely that the binding sites for the sesquiterpene lactones and BHQ on the ATPase involve not just the trans-membrane region of the protein.

Polar residues in helix M5 also appear to be important for helix packing. Single mutations S766A and S767A reduced the Ca²⁺ affinity of the ATPase [37, 38], whereas the mutation Y763F had no functional consequences [38]. However, the triple mutant Y763F, S766A, S767A showed no accumulation of Ca²⁺ (Table 1), was not phosphorylated by ATP in the presence of Ca²⁺ (Figure 3), but was phosphorylated by P_i in both the absence and presence of Ca²⁺ (Figure 5), suggesting that it was unable to bind

Ca²⁺. Thus these residues must be involved in the correct packing of helix M5 and formation of the two high-affinity Ca²⁺-binding sites on the ATPase.

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