Molecular characterization of a sarco(endo)plasmic reticulum Ca^{2+} -ATPase gene from *Paramecium tetraurelia* and localization of its gene product to sub-plasmalemmal calcium stores

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A cDNA encoding the gene for a sarco(endo)plasmic reticulum-type Ca^{2+} -ATPase (SERCA) was isolated from a cDNA library of *Paramecium tetraurelia* by using degenerated primers according to conserved domains of SERCA-type ATPases. The identified nucleotide sequence (PtSERCA) is 3114 nucleotides in length with an open reading frame of 1037 amino acids. An intron of only 22 nucleotides occurs. Homology searches for the deduced amino acid sequence revealed 38–49% similarity to SERCA-type ATPases from organisms ranging from protozoans to mammals, with no more similarity to some parasitic protozoa of the same phylum. The calculated molecular mass of the encoded protein is 114.7 kDa. It contains the typical 10 trans-membrane domains of SERCA-type ATPases and other con-

served domains, such as the phosphorylation site and the ATP binding site. However, there are no binding sites for phospholamban and thapsigargin present in the PtSERCA. Antibodies raised against a cytoplasmic loop peptide between the phosphorylation site and the ATP binding site recognize on Western blots a protein of 106 kDa, exclusively in the fraction of sub-plasmalemmal calcium stores ('alveolar sacs'). In immunofluorescence studies the antibodies show labelling exclusively in the cell cortex of permeabilized cells in a pattern characteristic of the arrangement of alveolar sacs. When alveolar sacs were tested for phosphoenzyme-intermediate formation a phosphoprotein of the same molecular mass (106 kDa) could be identified.

INTRODUCTION

A variety of cellular processes is regulated by calcium in many organisms ranging from protozoans to mammals. The current study considers importance of microdomain regulation of intracellular free Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$, in a variety of secretory systems [1]. Increasingly, relevance of sub-plasmalemmal calcium stores is inferred, e.g. from electrophysiological and Ca^{2+} -imaging studies, yet their identification has been elusive in most cases [1]. In *Paramecium* there are alveolar sacs attached directly to the plasma membrane [2]. These could be established as sub-plasmalemmal stores by different methods, e.g. Ca^{2+} -uptake studies after isolation [3,4], by Ca^{2+} localization via electron spectroscopic imaging [5] or secondary ion MS [6] and immunolocalization of a high-capacity/low-affinity Ca-binding protein [7].

In *Paramecium tetraurelia*, Ca^{2+} -regulated processes include exocytosis of trichocysts [2]. Trichocysts are secretory organelles whose contents can be extruded synchronously in response to natural stimuli, i.e. predators [8], or to artificial polyamino secretagogues [2]. Their expulsion is strictly dependent on calcium [2] and is accompanied by an increase of sub-plasmalemmal calcium concentrations to levels of several μM [9], whereby normally external sources and sub-plasmalemmal stores cooperate [5,6,9–12]. Trichocysts are attached to the cell membrane at genetically predetermined, regularly spaced, docking sites [2], each of which is located at the edges of four alveolar sacs [11], which may allow the cell to control tightly local $[\text{Ca}^{2+}]_i$ in the vicinity of docked trichocysts to prevent their constant firing and to rapidly establish $[\text{Ca}^{2+}]_i$ homeostasis after exocytosis induction. Alveolar sacs resemble sarcoplasmic reticulum of mammalian skeletal muscle, not only structurally [4], but also by their

calsequestrin content [7] and by sensitivity to caffeine [13]. It has also been shown that isolated alveolar sacs are able to pump calcium with strict dependence on ATP and Mg^{2+} [3,4]. Inhibitors of sarco(endo)plasmic reticulum Ca^{2+} -ATPases (SERCAs) of intracellular calcium stores cause reduced $^{45}\text{Ca}^{2+}$ -uptake into isolated alveolar sacs *in vitro* and trigger some exocytosis of trichocysts *in vivo*, yet only at unusually high concentrations [4]. Therefore, the question of the presence of a SERCA-type Ca^{2+} -ATPase in alveolar sacs has remained open so far.

We describe the isolation of a cDNA coding for a SERCA-type Ca^{2+} -ATPase from a *Paramecium* cDNA library. Peptide-specific antibodies (ABs) against a synthetic peptide of part of the deduced amino acid sequence ('C6 peptide') were used for subcellular localization studies. In Western blots, ABs recognize a protein with an apparent molecular mass of 106 kDa exclusively in the alveolar-sac fraction. Concomitantly in immunofluorescence labelling studies, ABs show a cortical staining pattern reflecting the arrangement of alveolar sacs. We also show the formation of a phosphoenzyme intermediate (EP) of 106 kDa in alveolar sacs dependent on the presence of Ca^{2+} and Mg^{2+} . Similarities of the deduced amino acid sequence of the SERCA gene from *Paramecium* to SERCA-type ATPases from closely related protozoa are low compared with the similarities of SERCAs from vertebrates to each other. This may explain the insensitivity of the protozoan SERCAs to SERCA inhibitors.

EXPERIMENTAL

Materials

Restriction enzymes came from New England Biolabs (Beverly, MA, U.S.A.), radioactive reagents from ICN Biomedicals

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; ABs, antibodies; CLSM, confocal laser-scanning microscopy; SERCA, sarco(endo)plasmic reticulum-type Ca^{2+} -ATPase; TG, thapsigargin; EP, phosphoenzyme intermediate.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number Y17496.

(Eschwege, Germany). All reagents and solvents used were of analytical grade.

PCR strategy

PCR was performed with 5 μ l (5×10^3 plaque-forming units) of a *P. tetraurelia* 51S cDNA library in λ ZAP Express (Stratagene GmbH, Heidelberg, Germany). Primers were designed to recognize the ATP phosphorylation site and the ATP-binding site of Ca^{2+} -ATPases of the SERCA type. It was also taken into account that *Paramecium* uses generally only A and T at the third position of a codon [14]. The sequences of the primers were: primer S1, 5'-TGYTCWGAYAAAGACTGGWAC-3' and primer S2, 5'-GGDGCATCRTTDACACCATC-3'. They were used in a final concentration of 0.625 μ M. PCR was carried out for 40 cycles for 45 s at 95 °C, 30 s at 46 °C and 1.5 min at 72 °C, with a final 10 min extension at 72 °C. The PCR products were cloned into the *EcoRV* restriction site of pBluescriptII SK(–). After sequencing of several clones, one clone named pSerca 1b was chosen for further investigations.

Screening of a λ ZAP Express cDNA library

Plaques (1×10^6) of a λ ZAP Express cDNA library of *P. tetraurelia* 51S from stationary phase were screened according to the instruction manual of Stratagene's λ ZAP Express Cloning Kit. The insert of pSerca 1b was amplified with primers S1 and S2 by PCR. The purified PCR fragment (50 ng) was labelled with 50 μ Ci of [α - 32 P]dCTP using the Rediprime DNA-labelling system from Amersham Life Science (Braunschweig, Germany) and used as a probe. Because of the high AT content of *Paramecium* DNA [14] hybridization was carried out at 38 °C in 50% formamide/0.1% (w/v) SDS/5 $\times$ SSC [150 mM NaCl/15 mM sodium citrate (pH 7.8)]/5 $\times$ Denhardt's solution [15]/100 μ g/ml denatured herring sperm DNA. Washing was in 0.1% (w/v) SDS/1 $\times$ SSC, twice for 10 min at 38 °C and twice for 30 min at 54 °C. The isolated positive plaques were screened in a second round as described above. Positive plaques were rescued by *in vivo* excision and sequenced. One clone was chosen for further studies. It was called clone C6.

Screening of a genomic DNA library

Primary plaques (3×10^5) of a λ ZAPII (Stratagene GmbH) genomic library of *EcoRI*-digested macronuclear DNA from *P. tetraurelia* 51S was screened as described above. The 470 bp *EcoRI* fragment of the cDNA clone C6 served as probe (see Figure 1B).

DNA sequencing

Plasmid DNA was purified by the Wizard Plasmid Miniprep DNA Purification System (Promega, Madison, WI, U.S.A.) and used as template for sequencing with the 32 Sequencing[®] Kit (Pharmacia, Uppsala, Sweden) and multiple primers (synthesized by MWG-Biotech GmbH, Ebersberg, Germany).

Computer analyses

Multiple sequence alignments were carried out by the Megalign program of the Lasergene Biocomputing Software (DNASTar Inc., Madison, WI, U.S.A.), using the Clustal method; pairwise alignments were done with the same program according to Lipman and Pearson [16]. FASTA homology searches were done at the FASTA3 server of the EMBL databases at Hinxton, Cambs., U.K. (<http://www2.ebi.ac.uk/fasta3/>).

Preparation of peptide-specific antibodies

Synthesis of the peptide SSQNEKGNVLFIKG (C6 peptide) and immunization was carried out by FZB Biotechnik GmbH, Berlin, Germany. Peptide was coupled to keyhole-limpet haemocyanin and injected into chicken. Immune serum was affinity purified against the C6 peptide.

Electrophoretic techniques and Western-blot analysis

SDS/PAGE and Western blots were carried out as described previously [17].

Cell fractionation

P. tetraurelia cells, strain 7S, were grown under sterile conditions and fractionated as follows. Cells were deciliated by a Ca^{2+} shock and cilia were purified by differential centrifugation [18]. Whole-cell homogenates were prepared in phase buffer [20 mM Tris-maleate/20 mM NaOH/20 mM NaCl/250 mM sucrose (pH 7.0)] by ≈ 100 hand strokes in a glass homogenizer equipped with a Teflon pestle. Soluble and particulate fractions were separated by centrifugation at 100 000 *g* for 60 min at 4 °C. Plasma-membrane vesicles were isolated according to Smith and Hennessey [19]. For preparation of sub-plasmalemmal calcium stores (alveolar sacs) and enriched microsomes, a combination of the isolation schemes of Wright and Van Houten [20], Stelly et al. [3] and Lange et al. [4] was used. Cells were suspended twice in ice-cold Deryl's solution [1 mM NaH_2PO_4 /1 mM Na_2HPO_4 /2 mM sodium citrate/1.5 mM CaCl_2 (pH 6.8)] to promote trichocyst discharge. Cells were then deciliated by incubation in an equal volume of STEN buffer [20 mM Tris/HCl/0.15 M sucrose/2 mM EDTA/6 mM NaCl (pH 7.5)] and, after 15 min on ice, 40 mM CaCl_2 was added. After 15–30 min on ice, deciliated cells were centrifuged, rinsed twice in ice-cold HM [20 mM Tris/HCl/1 mM EDTA/25 mM KCl/50 mM sucrose (pH 7.5)] and homogenized in a Thomas tissue grinder (size BB, 0.13–0.18 mm clearance; A. H. Thomas, Philadelphia, PA, U.S.A.) with 60–100 hand strokes. At this and the later steps, all at 0 °C, a protease-inhibitor cocktail (15 μ M pepstatin A/100 milliunits/ml aprotinin/100 μ M leupeptin/0.26 mM *p*-tosyl-L-arginine methyl ester/0.2 mM Pe-fabloc SC/28 μ M E64/16 μ M chymostatin/20 μ M antipain) was included. The homogenate was diluted with HM to 25 ml and centrifuged for 5 min at 3200 revs./min in a Sorvall JA 20 fixed-angle rotor. To obtain crude microsomes the supernatant was collected and processed as in [20]. The pellet containing alveolar sacs was diluted with HM to 10 ml, homogenized once again (≈ 20 hand strokes) and centrifuged for 5 min at 3200 revs./min in the same rotor. The pellet was resuspended in 25 ml of HMI [20 mM Tris/HCl/1 mM EDTA/25 mM KCl (pH 7.5)], and washed three times in the same buffer, each for 5 min at 2500 revs./min in a Heraeus Megafuge 1.0R, Sepatech 2150 swing-out rotor. After the final centrifugation step the pellet was suspended in 2–3 ml of HMI, homogenized once again by 3–5 strokes and aliquots of 150 μ l were layered on top of 10 ml transparent tubes containing HMI, 0.25 M sucrose and 22% (v/v) Percoll. Tubes were spun at 52 000 *g* in a Sorvall ultracentrifuge OTD Combi, Ti50 fixed-angle rotor (27 000 revs./min) for 20 min. The two upper bands were recovered by pipetting and pooled fractions were diluted in 20 volumes of HMI and centrifuged in a Sorvall JA 20 fixed-angle rotor for 10 min at 10 000 revs./min. The pellet was rinsed twice with the same buffer and twice with IK buffer [30 mM imidazole-HCl/100 mM KCl (pH 6.8)], each for 5 min at 2500 revs./min in a Heraeus Sepatech 2150, swing-out rotor. Finally, the pellets containing

predominantly alveolar sacs were suspended to a final volume of 1–2 ml at 0 °C.

EP analysis

Cell fractions were incubated with [γ - 32 P]ATP under conditions favourable for the formation of a stable phosphorylated-enzyme intermediate, as modified from Wuytack et al. [21]. Briefly, the reaction was started after 15 min of preincubation at 0 °C by adding 10 μ l of [γ - 32 P]ATP (3 nM at 4500 Ci/mmol) to 90 μ l of a medium containing IK buffer, freshly prepared alveolar sacs (5–10 μ g) in the presence or absence of 1 mM MgCl₂, 0.3 mM EGTA, 0.1 mM CaCl₂. The reaction was terminated after 20 s of incubation at 0 °C by adding 0.5 ml of ice-cold stop solution (10% trichloroacetic acid/50 mM phosphoric acid/0.5 mM ATP). After 10 min on ice, precipitated proteins were pelleted by centrifugation for 3 min in an Eppendorf centrifuge. This procedure was repeated three times before the pellets were rinsed with 0.5 ml ice-cold distilled water. Hydroxylamine sensitivity of the water-rinsed trichloroacetic acid precipitates was analysed in the presence of either 0.15 M sodium acetate/150 mM hydroxylamine hydrochloride (pH 6.0) or, for controls, 0.15 M sodium acetate/150 mM Tris/HCl (pH 6.0). After 10 min at 20 °C, the proteins were precipitated in stop solution, pelleted, rinsed with water and prepared for SDS/PAGE (samples were applied to SDS gels without boiling prior to electrophoresis).

Immunofluorescence labelling

Cells were fixed and permeabilized by successive treatment with acetone for 5 min, and then 4% formaldehyde in 0.1 M PBS for 10 min, both at 0 °C, followed by a wash in the same buffer supplemented with 1% (w/v) bovine serum albumin-C from BioTrend (Köln, Germany), incubation with primary ABs (see above), diluted 1:200, and FITC-coupled anti-chicken ABs (Sigma, Deisenhofen, Germany), diluted 1:50. After washing with PBS and covering by glycerol with 'Slow Fade' (Molecular Probes Inc., Eugene, OR, U.S.A.) added, evaluation was done by confocal laser-scanning microscopy (CLSM), using a Zeiss Axiovert microscope and a 100 mW Ar-laser (488 nm excitation) in combination with an Odyssey-LSM-C system from Noran (Bruchsal, Germany), recording at 515 nm in 0.18 μ m z-intervals.

Miscellaneous methods

Protein concentration was determined using BioRad protein-assay reagents. DNA concentrations were determined by measuring absorbance at 260 nm. Autoradiography was performed with dried silver-stained gels exposed to Amersham Hyperfilm-MP.

RESULTS

Isolation of a cDNA coding for a Ca²⁺-ATPase of the SERCA type

Ca²⁺-ATPases of the SERCA type are members of the P-type Ca²⁺ pumps, which contain several conserved domains, e.g. the ATP-binding site and the phosphorylation site (reviewed in [22]). Degenerated oligonucleotides (double underlined in Figure 1A) were synthesized accordingly. Primer S1 was synthesized according to amino acids Cys³⁵⁸–Thr³⁶⁴ of rat SERCA 2A [23] and primer S2 according to amino acid Asp⁷²¹–Pro⁷²⁷. For the degeneration of the two primers the *Paramecium* codon-usage frequency was calculated from already sequenced genes of

Table 1 Comparison of the deduced amino acid sequence of SERCA-type ATPase from *P. tetraurelia* with other SERCA sequences

Pairwise percentage similarities are shown, calculated by the method of Lipman and Pearson [16] (Ktuple = 2, gap penalty = 4 and gap-length penalty = 12). Pt, *P. tetraurelia*; Rb, rabbit isoform 2 [31]; Dm, *Drosophila melanogaster* [44]; Af, *Artemia franciscana* [45]; Pf, *Plasmodium falciparum* [46]; Lm, *Leishmania mexicana* [37]; Tb, *Trypanosoma brucei* [33]; Sm, *Schistosoma mansoni* [47]; Tv, *Trichomonas vaginalis* [38]; At, *Arabidopsis thaliana* [29].

	1	2	3	4	5	6	7	8	9	10
	Pt	Rb	Dm	Af	Pf	Lm	Tb	Sm	Tv	At
1 <i>P. tetraurelia</i>	–	45.9	45.8	45.2	45.3	40.7	41.7	45.4	38.5	49.1
2 Rabbit	–	–	72.1	71.6	43.6	44.6	47.7	67.7	41.5	48.6
3 <i>D. melanogaster</i>	–	–	–	79.7	41.8	44.7	48.8	67.5	40.9	48.9
4 <i>A. franciscana</i>	–	–	–	–	45.0	44.9	47.1	66.0	42.9	48.9
5 <i>P. falciparum</i>	–	–	–	–	–	38.2	40.0	46.0	38.6	47.9
6 <i>L. mexicana</i>	–	–	–	–	–	–	63.2	45.6	35.5	44.2
7 <i>T. brucei</i>	–	–	–	–	–	–	–	47.9	36.8	43.1
8 <i>S. mansoni</i>	–	–	–	–	–	–	–	–	39.5	48.7
9 <i>T. vaginalis</i>	–	–	–	–	–	–	–	–	–	39.8
10 <i>A. thaliana</i>	–	–	–	–	–	–	–	–	–	–

Paramecium ([17,24,25] and Accession numbers U31445 and U19464). We also have taken into account the AT richness of *Paramecium* coding DNA [14] and the fact that *Paramecium* is more likely to have an A or a T at the third position of a codon. These oligonucleotides were used to amplify a PCR product from a λ ZAP Express cDNA library from *P. tetraurelia* cells. The amplified fragment was about 1.1 kb in length and subcloning and sequencing showed that it covered one third in the middle of the expected open reading frame (Figure 1B). It was used as a probe to screen the same λ ZAP Express cDNA library. Three rounds of screening resulted in 11 independently derived clones. Four of them contained an insert of $\approx$ 3 kb. Sequencing showed that they belonged to two groups of closely related cDNAs. The search for open reading frames revealed that they were all lacking the start codon, ATG. One of them, clone C6, was chosen for further investigation and sequenced completely (Figure 1A).

To complete the missing 5' end of the isolated cDNA, a genomic λ ZAPII library of *P. tetraurelia* was screened with the 470 bp *Eco*RI fragment of C6, which covers the first 470 bp of the 5' end of the open reading frame in clone C6 (Figure 1B). Fifteen independent clones were derived after the third round of screening. Three of them were sequenced and they all contained the correct 5' end with the start codon ATG and another 209 bp 5'-untranslated sequence. The genomic fragment contains a short intron of 22 nucleotides the length of which is typical of *Paramecium* [26] (Figure 1A).

SERCA cDNA and its deduced amino acid sequence

The open reading frame of the isolated SERCA cDNA consists of 3114 nucleotides (Figure 1A). The assigned start ATG in Figure 1(A) is assumed to be the correct start codon of the coding region since there is a stop triplet, TGA, 37 codons upstream in the reading frame. The next in-frame ATG is at nucleotide position #406 (amino acid #136). It follows amino acid sequences that align to conserved membrane-spanning sequences of membrane domains M₁ and M₂ and, therefore, cannot be considered as the start codon for this gene. There is little coincidence with

A Phosphorylation site

<i>P. tetraurelia</i>	306	KVAVALAVAAIPEGLPAVITTCALGARRMAKQKAIV--RKLPKVOTLG-CTTIIICSDKTGTLTTNEMC
Rabbit SERCA2B	297	KIAVALAVAAIPEGLPAVITTCALGTRRRMAKNAIV--RSLPSVETLG-CTSVICSDKTGTLTTNQMS
<i>D. melanogaster</i>	297	KIAVAVAVAAIPEGLPAVITTCALGTRRRMAKNAIV--RSLPSVETLG-CTSVICSDKTGTLTTNQMS
<i>A. franciscana</i>	300	KIAVALAVAAIPEGLPAVITTCALGTRRRMAKNAIV--RSLPSVETLG-CTSVICSDKTGTLTTNQMS
<i>P. falciparum</i>	314	KISVALAVAAIPEGLPAVITTCALGTRRMVKKNAIVFTRKQSVETLG-CTTVICSDKTGTLTTNQMT
<i>L. mexicana</i>	313	KVAVALAVAAIPEGLPAVITTCALGARKMARHNAIV--RDLPSVETLGRCT-VICSDKTGTLTTNMMS
<i>T. brucei</i>	303	KVAVALAVAAIPEGLPAVITTCALGTRRMAQHNAIV--RDLPSVETLGRCT-VICSDKTGTLTTNMMS
<i>S. mansoni</i>	298	KIAVALAVAAIPEGLPAVITTCALGTRRRMAKNAIV--RSLPSVETLG-CTTVICSDKTGTLTTNQMT
<i>T. vaginalis</i>	295	KIAISLAVAAIPEGLPAVITMTLSLGVNRMAKNAIV-TK-LPAVETLG-CTSVICSDKTGTLTTNKMV
<i>A. thaliana</i>	329	ETAVVALAVAAIPEGLPAVITTCALGTRKMAQKNAIV--RKLPVETLG-CTTVICSDKTGTLTTNQMA

B FITC-binding site/T₁ tryptic site

<i>P. tetraurelia</i>	484	-NEFAK-RA-TLEFTRDRKSMVS-----LASSQNEKGNVLF- IK GAPEYILLEKST
Rabbit SERCA2B	480	-----K-KEFTLEFSRDRKSMVSICYT-----PNKPSRTSMS-K-----MF-VKGAPEGVDRCT
<i>D. melanogaster</i>	480	-----K-KEFTLEFSRDRKSMVSICYT-----PLKASRLGTGPK-----LF-VKGAPEGVDRCT
<i>A. franciscana</i>	484	-----K-KEFTLEFSRDRKSMVSICYV-----PLKAGLLSNGPK-----MF-VKGAPEGVDRCT
<i>P. falciparum</i>	708	-KQI-KIIEFT--FTRERKLSMV-----IVEN-----KKKEIILY-CKGAPENIKNCK
<i>L. mexicana</i>	495	-----L-KKATLEFTRSRKSMVCCSTST-----RHS---LF-VKGAPEEILKRC
<i>T. brucei</i>	478	-----K-KNATLEFTRKRSMSVHTSTVTGSP-----ASSTNN---LF-VKGAPEEVLRR-S
<i>S. mansoni</i>	485	-----E-RKFTLEFSRDRKSMSTYVI-----P--QSQIS-GSKEK-----LF-VKGAPESIDRCT
<i>T. vaginalis</i>	473	YKEYPKVR--THEFTRARKSMS--C-----IVGK-----NTL-VKGAPEVILAKCD
<i>A. thaliana</i>	500	-SELEQ-RIATLEFDRDRKSMGV-----MVDSSSGNK-----LLLKGAENVLERST

C Membrane spanning domain III (thapsigargin binding)

<i>P. tetraurelia</i>	257	DPLSKRLDEFGDKLAKYVT---YIGIICWV--MNIGNESDPAY
Rabbit SERCA2B	247	TPLQOKLDEFGQLSKVIS---LIGIAVWI--INIGHFNDPVH
<i>D. melanogaster</i>	247	TPLQOKLDEFGQLSKVIS---VICAVVA--INIGHFNDPAH
<i>A. franciscana</i>	250	TPLQOKLDEFGQLSKVIS---VICAVVA--INIGHFNDPAH
<i>P. falciparum</i>	263	TPLQIKIDLFGQLSKIIF---VICVTWIFTINFKHESDPIH
<i>L. mexicana</i>	256	TPLQKLDEFGVLLSGVIG---YICLFVFW--ANL-----L
<i>T. brucei</i>	246	TPLQVKLDEFGVLLSKVIG---YICLVVFA--VNL-----V
<i>S. mansoni</i>	248	TPLGQKIDFEGTQLSKVIT---FICIAVWC--INIGHFNDPVH
<i>T. vaginalis</i>	246	TPLQRLDNE---SKYISVGILFICVITW-FA-NISKDE-VG
<i>A. thaliana</i>	272	TPLKKKLNEFGEVLTMIIG---LICALVWL--INVKYF-----L

Figure 2 Alignment of three domains of the *P. tetraurelia* SERCA-type ATPase with known SERCA sequences

(A) Phosphorylation site. Indicated in black is the aspartyl residue that is phosphorylated during calcium transfer. (B) FITC-binding site/T₁ tryptic site. Shown in black is the lysine residue that binds FITC. The Arg³⁰⁵ in rabbit SERCA-2B sequence (black bar), the cleavage site T₁ for trypsin, is also indicated. The boxed amino acid sequence in the *P. tetraurelia* sequence indicates the oligopeptide used for immunization to raise peptide-specific Abs. (C) Shows a comparison of the membrane-spanning domain M₃ (boxed), which contains the sequences essential for thapsigargin (TG) binding [34]. Underlined sequences belong to SERCA-type ATPases, which so far are reported not to be inhibited by TG (see text). The only SERCA-type ATPase in this Figure known to be inhibited by TG is rabbit SERCA-2B.

consensus sequences usually found in eukaryotic promoters such as TATA, CAT or GC boxes. The sequence ⁻³ATAATGG⁺⁴ around the start ATG might have similarity to the sequence ⁻³AXYATGG⁺⁴, found by Kozak [27], typical of the environment of the start ATG in eukaryotic genes. The 3'-non-coding region contains a poly(A⁺) tail. The GC content of the coding region is 36.2 % (63.8 % AT). In the non-coding region the GC content is only 19.3 % (80.7 % AT).

The deduced amino acid sequence is 1037 amino acids in length, with a calculated molecular mass of 114.7 kDa. The amino acid sequence contains 44 glutamine residues. Ten residues are encoded by the glutamine codon CAA and none by the codon CAG. The remaining 34 residues are encoded by the universal termination codons TAA (28 residues) or TAG (6 residues), which are the preferred codons for glutamine in *Paramecium* [28].

A FASTA homology search in the Swissprot protein database revealed 49.1 % similarity over 995 amino acids to the SERCA-type Ca²⁺-ATPase from *Arabidopsis* [29] and 38.5–45.9 % similarity to SERCAs from other species ranging from protozoans to mammals (Table 1). It shares only 25 % identity with

plasmalemmal Ca²⁺-ATPase from *Paramecium* [30]. Analysis of the deduced amino acid sequence shows that this gene product contains all the conserved domains typical of SERCA-type Ca²⁺-ATPases (reviewed in [22]). The phosphorylation domain contains the phosphate acceptor Asp³⁶⁰ (corresponding to Asp³⁵¹ in rabbit skeletal muscle SERCA-2 [31]; Figure 2A), which is phosphorylated by ATP during Ca²⁺ transport. The FITC-binding site Lys⁵¹⁸ (Figure 2B) and the reaction site Lys⁷⁰¹ in the nucleotide-binding domain are also present. The high-affinity calcium-binding site is presumably located in the transmembrane helices M₄, M₅, M₆ and M₈ since they contain the important amino acids Glu³¹⁸, Glu⁷⁹⁰, Asn⁸¹⁵, Thr⁸¹⁸, Asp⁸¹⁹ and Glu⁹⁴² (Figure 1A) [22]. The hydropathy plot of the deduced amino acid sequence (not shown) yields a profile containing 10 transmembrane domains (underlined in Figure 1A), as expected for SERCA-type ATPases [22]. There is no calmodulin-binding domain at the C-terminal end of the PtSERCA polypeptide. The phospholamban-binding motif KDDKPV, typical of SERCA-2 [32], is also not present. There is also no thapsigargin-binding consensus sequence [33,34] to be found in the PtSERCA protein sequence (Figure 2C).

Figure 3 Western blot containing different cell fractions

100 000 *g* pellet (lane 1, pe), alveolar-sac fraction (lane 2, as), microsomal fraction (lane 3, mi), plasma-membrane vesicles (lane 4, pm) and cilia (lane 5, ci). Each lane was loaded with 50 μ g of protein. The blot was probed with peptide-specific ABs against C6 peptide. The arrow indicates the protein band at 106 kDa in alveolar-sac fraction.

Figure 4 CLSM immunofluorescence labelling with ABs against C6 peptide

(A) Superficial view, (B) median view. Note the coincidence of labelling pattern with the linear cortical arrangement of alveolar sacs of about 1–2 μ m size. Scale bars, 10 μ m.

Localization of SERCA

For localization studies, peptide-specific ABs against PtSERCA were raised in chicken. The deduced amino acid sequence of PtSERCA was analysed by the Protean program of the LASERGENE DNA software package to search for surface probability and antigenicity of partial sequences within the PtSERCA amino acid sequence. We chose the peptide SSQ-NEKGNVLFKIG (amino acids #506–519; C6 peptide) because of its high antigenicity index and surface probability (results not shown). It is located between the phosphorylation domain and the nucleotide-binding domain within a stretch that shows no similarity to corresponding regions in other SERCAs (Figure 2B). In SERCA-2 of rabbit skeletal muscle, in this region the

Figure 5 EP-formation autoradiography in isolated alveolar sacs

Freshly prepared alveolar sacs were incubated for 20 s at 0 °C with [γ -³²P]ATP with the following additions, as indicated: 0.1 mM CaCl₂, 0.3 mM EGTA, 1 mM MgCl₂. Acid-precipitated aliquots of alveolar sacs (8.5 μ g) were subjected to SDS/PAGE under Laemmli conditions and processed for autoradiography. Note that under standard conditions (lane 1), Ca²⁺-pump-specific EP forms only at 106 kDa (arrow) in a strongly Ca²⁺- (lane 2) and Mg²⁺- (lane 3) dependent manner.

tryptic cleavage site T₁ is located [22]. It is missing from the *Paramecium* SERCA polypeptide. Figure 3 shows a typical Western blot containing different subcellular fractions of *Paramecium* cells probed with affinity-purified polyclonal ABs against C6 peptide. The antibody recognizes a single protein band of $\approx$ 106 kDa only in the alveolar-sac fraction, but not in other cell fractions.

Figure 4 shows a CLSM immunofluorescence micrograph of a *Paramecium* cell labelled with ABs against C6 peptide, visualized by FITC. The upper panel shows the labelling of the cell surface in *en face* view. Labelling pattern reflects the typical regular arrangement of alveolar sacs, about 1–2 μ m in size. The lower panel shows labelling in the median focus, thus demonstrating restriction of the label to the cortex of the cell. No label occurred without primary AB. Controls with non-permeabilized cells were also to the negative.

Demonstration of an EP in alveolar sacs

Since all P-type Ca²⁺-transport ATPases transiently form a characteristic Ca²⁺-dependent EP with hydroxylamine-labile acyl bonds [35], we analysed this aspect in freshly prepared alveolar-sac fractions using well-defined assay conditions for mammalian Ca²⁺-ATPases of the SERCA type [21]. In the presence of [γ -³²P]ATP, MgCl₂ and CaCl₂ phosphorylation can be visualized as a ³²P-labelled 106 kDa protein in autoradiograms, not only on acidic gels (results not shown), but also on Laemmli gels (Figure 5). Since this ³²P labelling is strongly sensitive to hydroxylamine (results not shown) and also depends on the presence of Ca²⁺ and Mg²⁺ (Figure 5, lanes 2 and 3) we assume the 106 kDa protein to be the EP of the Ca²⁺-ATPase in alveolar sacs.

DISCUSSION

In its cortex *Paramecium* possesses a vast calcium-storage compartment, the so-called alveolar sacs (see Introduction). These flat sacs are physically attached to the plasma membrane, thus showing some resemblance to the sarcoplasmic reticulum of mammalian muscle cells [4]. Their ability to actively pump Ca^{2+} [3,4] led us to analyse whether they contain a Ca^{2+} -ATPase of the SERCA type. Here we report the isolation of a gene from a cDNA library of *Paramecium* which codes for a Ca^{2+} -ATPase of the SERCA-type and we show that the product of this gene is located exclusively in alveolar sacs. We also show the formation of a typical EP in these calcium stores. Therefore, we assume that this SERCA-type ATPase is the ATPase responsible for Ca^{2+} uptake into the alveolar sacs.

Aspects pertinent specifically to *Paramecium*

The nucleotide sequence of the PtSERCA gene, with an open reading frame of 3114 nucleotides, shows typical features of *Paramecium* genes, i.e. it is very AT rich, the coding region containing 63.8% AT, the non-coding region 80.7%. The codon-usage frequency also mirrors the AT richness of the DNA, since 70% of the codons end with an A or T and only 10 of the 44 glutamine residues are encoded by CAA (there occurs no CAG), whereas the residual 34 glutamine codons are the universal termination codons TAA (28 residues) or TAG (6 residues), according to the aberrant genetic code of ciliates [28]. As already found with other *Paramecium* genes [17,25,30] there are no usual promoter sequences present, such as CAAT, GC or TATA boxes. There might be a distant similarity with the consensus sequence found by Kozak [27] typical of the sequence around the start ATG (see Results). However, this sequence is obviously not necessary for recognition of the transcription start site since it is not present in several other *Paramecium* genes investigated so far [17,24]. We also found a short intron of 22 nucleotides in the part of the genomic sequence of PtSERCA that was analysed. The short length of the intron is typical of *Paramecium* [26].

Comparison with other Ca^{2+} pumps

The deduced amino acid sequence of the open reading frame of the PtSERCA gene consists of 1037 amino acids and has a calculated molecular mass of 114.7 kDa. Homology searches in the databases show a 40–50% overall similarity with SERCA-type ATPases (Table 1), and partial similarities over conserved regions of 23–32% to other P-type ATPases in general. A multiple sequence alignment (results not shown) with other SERCA sequences (for references see Table 1) shows conservation of all important domains of Ca^{2+} -ATPases (as reviewed in [22]), such as the phosphorylation site (Figure 2A), nucleotide-binding site and several conserved amino acids essential for Ca^{2+} binding and transport (Figure 1A). Surprising is the fact that SERCAs from evolutionarily closely related organisms, such as *Plasmodium*, *Leishmania* and *Trypanosoma*, do not show more similarity to one another than to other organisms, though they all belong to the phylum Alveolata and they all are thapsigargin (TG) insensitive (see below). Since this is the first analysis of a SERCA in alveolar sacs, localization to distinct stores of the products coded by published SERCA genes from parasitic members of the Alveolata now appears advisable.

The amino acid sequence of PtSERCA is clearly distinguished from that of the plasma membrane Ca^{2+} -ATPase. Plasma membrane Ca^{2+} -ATPases have an apparent molecular mass of ≈ 130 kDa, which is due to a C-terminal elongation of ≈ 200 amino acids containing a calmodulin-binding site [35,36]. This

domain is completely missing in the PtSERCA polypeptide in agreement with the apparent molecular mass of 106 kDa of the PtSERCA gene product (Figure 3). The SERCA-type ATPase of *Paramecium* is not regulated by calmodulin (R. Kissmehl, unpublished work). These results are also supported by recent cloning of the gene for the plasma-membrane Ca^{2+} -ATPase in *P. tetraurelia* [30], the deduced amino acid sequences of PtSERCA and this plasmalemmal Ca^{2+} -ATPase showing only 25% similarity.

Functional domains and subcellular localization

Vertebrate SERCA-type ATPases can be specifically inhibited by TG, which binds to sequences located within transmembrane domain M_3 [34]. In the PtSERCA polypeptide the sequence within the transmembrane domain M_3 shows little similarity to the corresponding sequence in rabbit SERCA-2B (Figure 2C). This is also the case for SERCA-type ATPases from *Leishmania mexicana* [37], *Arabidopsis thaliana* [29] and *Trichomonas vaginalis* [38] (Figure 2C). For the Ca^{2+} -ATPases from *L. mexicana* and *A. thaliana* it has been shown that they are not inhibited by TG, which is in agreement with the lack of a TG-binding site. The same TG insensitivity has been observed in biochemical characterization of the Ca^{2+} -ATPase in alveolar sacs from *Paramecium* [4]. Additionally we found that TG does not influence formation of a 106 kDa EP characteristic of Ca^{2+} -ATPases in alveolar sacs (R. Kissmehl, unpublished work). Sorin and co-workers have recently described a third type of Ca^{2+} -ATPase located in the Golgi apparatus that is also not inhibited by TG [39]. However, the PtSERCA described here is different from this Ca^{2+} -ATPase, since the two proteins show only 21.9% overall similarity to each other, compared with 41% overall similarity between PtSERCA and rabbit SERCA-2B.

The lack of a phospholamban-binding site KDDKPV [32] is not surprising since it is only present in mammalian SERCA-2 and SERCA-1 Ca^{2+} -ATPases but lacking in all other SERCA-type ATPases of higher eukaryotes [40]. There is also no evidence for the presence of phospholamban in *Paramecium* (R. Kissmehl, unpublished work).

As already mentioned the PtSERCA polypeptide has a calculated molecular mass of 114.7 kDa. A specific AB against a synthetic oligopeptide of the PtSERCA sequence recognizes a band of 106 kDa on Western blots (Figure 3). The difference between the apparent and the calculated molecular masses is not unusual for SERCA-type ATPases. It has been shown for the *Arabidopsis* SERCA-type ATPase (106/116 kDa) [29], the human SERCA-3 ATPase (97/109 kDa) [41], SERCA-2B ATPase (104/115 kDa) [42] and SERCA-2A ATPase (100/110 kDa) [43] and is very likely due to its hydrophobic character. The immunoreactive band of 106 kDa occurs only in the alveolar-sac fraction of *Paramecium* cells but not in cilia or plasma-membrane fractions. There is also no signal in the 100 000 g pellet, probably due to the low relative amount of PtSERCA peptide present. The exclusive cortical localization in CLSM immunofluorescence selectively in permeabilized cells (Figure 4) further supports the occurrence of the PtSERCA gene product in alveolar sacs, particularly since the labelling pattern coincides with the arrangement of alveolar sacs (see Introduction). Immunolabelling at the electron-microscopic level also shows that the PtSERCA gene product is located in the alveolar-sac membranes and not in the plasma membrane (M. Momayezi and H. Plattner, unpublished work).

Formation of a hydroxylamine-labile EP in alveolar sacs also yields a phosphoprotein of 106 kDa (Figure 5), which agrees with the size of the protein recognized by peptide-specific ABs

against the gene product of PtSERCA. The formation of EP is dependent on Ca^{2+} and Mg^{2+} at 0 °C during brief times, thus distinguishing it from potential kinase substrates [21]. This conforms with a similar reaction mechanism accepted for all Ca^{2+} -sequestering ATPases analysed so far.

Conclusion

This report of a Ca^{2+} -ATPase of the SERCA type in alveolar sacs is the first description of an organellar Ca^{2+} -ATPase in *Paramecium*. Whether there are other Ca^{2+} pumps present in the endoplasmic reticulum or the Golgi apparatus remains to be investigated.

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