Subplasmalemmal Ca-stores in *Paramecium tetraurelia*. Identification and characterisation of a sarco(endo)plasmic reticulum-like Ca^{2+} -ATPase by phosphoenzyme intermediate formation and its inhibition by caffeine

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Summary Considering increasing interest in calcium stores in protozoa, including parasitic forms, and specifically in subplasmalemmal stores in higher eukaryotes, we have isolated subplasmalemmal calcium stores (alveolar sacs) from the ciliated protozoan, *Paramecium tetraurelia*. Using antibodies against established sarco(endo)plasmic reticulum Ca^{2+} -ATPases (SERCAs) we detected in Western blots of subcellular fractions a band of ~106 kDa size selectively in alveolar sacs – but not, for example, in plasma membranes – and concomitant restriction of immunofluorescence labelling to the cell cortex of permeabilised cells. These results are the same as with ABs against a peptide derived from a cloned SERCA-like gene from *Paramecium* [Hauser K., Pavlovic N., Kissmehl R., Plattner H. Molecular characterization of a sarco(endo)plasmic reticulum Ca^{2+} -ATPase gene from *Paramecium tetraurelia* and localisation of its gene product to subplasmalemmal calcium stores. *Biochem J* 1998; **334**: 31–38]. When such isolated alveolar sacs were now tested for phosphoenzyme intermediate (EP) formation, a phosphoprotein of the same apparent molecular mass (~106 kDa) as in blots could be identified in gel autoradiograms. This EP corresponds to that formed in the reaction cycle of different SERCA-types, with dependency on Ca^{2+} and Mg^{2+} , sensitivity to La^{3+} or insensitivity towards calmodulin, calmodulin antagonists and vanadate. However, EP formation in alveolar sacs is not inhibited by established SERCA inhibitors (e.g. thapsigargin tested up to 100 μM). Surprisingly, caffeine, which is frequently used to mobilise Ca^{2+} from intracellular stores, strongly inhibits EP formation. In parallel experiments, we did not find any similar effect with sarcoplasmic reticulum isolated from skeletal muscle. We conclude that the ~106 kDa protein of alveolar sacs in *Paramecium* may represent a SERCA-like Ca^{2+} -ATPase with some unorthodox features, which might be relevant also for some other protozoan systems. In this case, the established Ca^{2+} -mobilizing effect of caffeine may be amplified by inhibiting store refilling.

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INTRODUCTION

Calcium plays a pivotal role in the regulation of many cellular processes, not only in mammalian, but also in protozoan, cells. In *Paramecium* cells this encompasses mating, ciliary beating, chemotaxis, and exocytosis

(reviewed in [1]). Most of their secretory organelles ('trichocysts') are docked at the cell surface ready for synchronous release [2]. Stimulation results in a rapid subplasmalemmal $[Ca^{2+}]_i$ increase even in the absence of extracellular Ca^{2+} ($[Ca^{2+}]_o$) [3]. This may be due to the occurrence of vast subplasmalemmal calcium stores, the 'alveolar sacs' [4–6] which are tightly attached at the cell membrane, with each trichocyst positioned at the edge of four adjacent sacs [7]. Alveolar sacs may be a special form of endoplasmic reticulum (ER) sharing several characteristics with mammalian sarcoplasmic reticulum (SR) [8], including the existence of a calsequestrin-like high capacity/low affinity Ca^{2+} -binding protein [9]. They also show strong sensitivity to caffeine [10,11], frequently used up to 50 mM in various cell systems to mobilise Ca^{2+} from intracellular stores via ryanodine-sensitive Ca^{2+} channels [12–15]. However, there is no evidence for a ryanodine sensitive release mechanism in *Paramecium* [8,16]. Caffeine may also act as an inhibitor of a Ca^{2+} -ATPase [10] whose occurrence in isolated alveolar sacs has been implied from $^{45}Ca^{2+}$ sequestration studies [4,8]. This Ca^{2+} -ATPase is believed to be related to a sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA)-type [8] which is like the plasmalemmal Ca^{2+} -pump, a P-type transport ATPase characterised by the transfer of the γ -phosphate of ATP to the enzyme to form a transient phosphoenzyme intermediate [17–19]. Although the reaction mechanism of the two types of Ca^{2+} pumps is essentially identical, involving the transient formation and hydrolysis of an acylphosphate bond to an aspartate residue, SERCAs strongly differ from plasmalemmal Ca^{2+} pumps in several characteristics, e.g. molecular mass, immunological reactivity, sensitivity towards pharmacological agents or in the differential interaction with regulator proteins [17–19]. In mammalian cells, several SERCA isoforms have been identified that are derived from three genes, SERCA-1, SERCA-2 and SERCA-3, which each has a distinct pattern of tissue-specific and developmentally regulated expression [19]. Their molecular masses differ between 97 and 116 kDa depending on the isoform, tissue and species. However, additional structurally distinct types exist [20] suggesting that the full extent of heterogeneity among SERCA-type Ca^{2+} pumps has not yet been fully explored. Nevertheless, we could use antibodies (ABs) against established SERCAs and against a peptide derived from the cloned gene of the Ca^{2+} -ATPase precisely of this subplasmalemmal calcium pool in *Paramecium* (see Results and Discussion) as specific probes.

The main problems in our work were to: (i) discriminate between Ca^{2+} -ATPases in the plasma membrane and in tightly attached stores; and (ii) account for unexpected drug effects. In the present study, we have taken advantage of some characteristic properties of

mammalian SERCA-type Ca^{2+} -ATPases with regard to formation of phosphoenzyme intermediates for their identification in subplasmalemmal calcium stores of *Paramecium*. These thapsigargin (TG)-insensitive stores in part share some characteristics with, but in part are different from, established mammalian SERCAs. A novel aspect is inhibition by caffeine.

Our findings might be of more general interest, considering the importance of Ca^{2+} for host cell invasion by parasitic protozoa [21], which, like *Paramecium*, belong to the phylum of 'alveolata' [22] and the involvement of ill-defined but frequently postulated, subplasmalemmal Ca^{2+} stores in exocytosis, up to mammalian systems [23–25].

MATERIALS AND METHODS

Materials

2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (TBHQ, 99%) was from Aldrich (Steinheim, Germany). Cyclopiazonic acid (CPA), thapsigargin (TG), caffeine, aprotinin, ruthenium red, sodium orthovanadate, N-*p*-tosyl-L-arginine methyl ester (TAME) and E64 were obtained from Sigma. Compound R24571 (calmidazolium), thapsigargin (TGC), chymostatin and antipain were from Calbiochem (San Diego, CA, USA). Protein assay dye reagent was purchased from Bio-Rad (Munich, Germany), trifluoperazine (TFP) from Boehringer-Mannheim and pepstatin A from Serva (Heidelberg, Germany). Pefabloc SC and leupeptin were from Biomol (Hamburg, Germany), SDS protein molecular weight markers (LMW and HMW) from Pharmacia LKB Biotechnology and nitrocellulose (NC)-membranes (BA-85) were from Schleicher-Schuell (Dassel, Germany). Radioactive $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ was from ICN Biomedicals (Eschwege, Germany). All solvents and other reagents used were of analytical grade.

Cell culture and cell fractionation

Paramecium tetraurelia wild type cells (strain 7S) were grown sterilely at 25°C to early stationary phase as previously described [26]. Cells were deciliated by a Ca^{2+} -shock and cilia were purified by differential centrifugation [27]. 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 homogeniser equipped with a Teflon pestle. Soluble and particulate fractions were separated by centrifugation at 100,000 g for 60 min at 4°C. Cell surface complexes ('cortices') were prepared as in Lumpert et al [28]. Trichocysts were isolated by the

method of Glas-Albrecht and Plattner [29] and plasma membrane vesicles according to Smith and Hennessey [30]. For preparation of subplasmalemmal calcium stores ('alveolar sacs') and enriched microsomes, a combination of the isolation schemes of Wright and Van Houten [31], Stelly et al [4] and Lange et al [8] was used. Briefly, cells were suspended twice in ice-cold Dryl's solution (1 mM NaH_2PO_4 , 1 mM Na_2HPO_4 , 2 mM Na_3 -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 homogenised in a Thomas tissue grinder (size BB, 0.13–0.18 mm clearance; A.H. Thomas, Philadelphia, PA, USA) with 60–100 hand strokes. At this and the later steps, all at 0°C, a protease inhibitor cocktail was included (see below). The homogenate was diluted with HM to 25 ml and centrifuged for 5 min at 3200 rpm in a Sorvall JA20 fixed angle rotor. To obtain crude microsomes the supernatant was collected and processed as described by Wright and Van Houten [31]. The pellet containing alveolar sacs was diluted with HM to 10 ml, homogenised once again (~20 hand strokes) and centrifuged for 5 min at 3200 rpm 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 3 times in the same buffer, each for 5 min at 2,500 rpm 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, homogenised once again by 3–5 strokes and aliquots of 150 μl 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 rpm) 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 JA20, fixed angle rotor, for 10 min at 10,000 rpm. 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 rpm 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. SR was prepared from white portions of psoas muscles from adult rabbits containing SERCA-1 [32]. A protease-inhibitor cocktail of 15 μM pepstatin A, 100 mU/ml aprotinin, 100 μM leupeptin, 0.26 mM TAME, 0.2 mM Pefabloc SC, and, in some cases for alveolar sac preparations, additionally 28 μM E64, 16 μM chymostatin and 20 μM antipain was used.

Phosphoenzyme intermediate analysis

In order to identify Ca^{2+} -stimulated ATPase from intracellular calcium stores, 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 [33]. Briefly, the reaction was started after 15 min of pre-incubation at 0°C by adding 10 μl [γ - ^{32}P]-ATP (3 nM at 4500 Ci/mmol) to 90 μl of a medium containing IK buffer, freshly prepared alveolar sacs (5–10 μg) or SR from rabbit skeletal muscle (8 μg) in the presence or absence of 1–4 mM MgCl_2 , 0.3 mM EGTA, 50 μM LaCl_3 , CaCl_2 , caffeine and various SERCA inhibitors (TG, TGC, CPA, TBHQ) in concentrations up to 100 μM as indicated. CaM-dependency was analysed in the presence or absence of either 2 μM *Paramecium* CaM, 30 μM ruthenium red, 30 μM sodium vanadate and 100 μM TFP or calmidazolium. Pre-incubation with $\text{Ca}^{2+}/\text{Mg}^{2+}$ was either before or after adding potential effectors. 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 [TCA], 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 3 times before the pellets were rinsed with 0.5 ml ice-cold distilled water. Hydroxylamine sensitivity of the water-rinsed TCA precipitates was analysed in the presence of either 0.15 M NaOAc plus 150 mM hydroxylamine hydrochloride, pH 6.0 or, for controls, in 0.15 M NaOAc plus 150 mM Tris-HCl, pH 6.0 [33]. After 10 min at 20°C, the proteins were precipitated in stop solution, pelleted, rinsed with water and prepared for SDS-PAGE.

Electrophoretic techniques

Phosphorylated samples were subjected to SDS-PAGE either under acidic conditions [33] or using the conditions of Laemmli, but without boiling samples prior to electrophoresis. Non-phosphorylated samples of the various cell fractions were also separated on linear gradient (5–10%) SDS polyacrylamide gels. Gels were either stained with silver or prepared for blotting onto nitrocellulose membranes as in [34].

Western blots

Western blot analysis was performed according to Kissmehl et al [34] using polyclonal antisera, either against SERCA-2A [35] or against a peptide (aa506–519) from a recently cloned SERCA-type gene from *Paramecium* [36].

Immunofluorescence labelling

Cells were fixed and permeabilised as in Plattner et al [9] and incubated with SERCA specific antibodies (ABs)

Fig. 1 Phase contrast micrograph of isolated alveolar sacs from *P. tetraurelia*. Note that the alveolar system is composed of a mosaic of flattened membrane-bounded sacs which may be joined laterally. Magnification 1000 $\times$.

diluted 1:40 in PBS plus 1% BSA for 60 min at 20°C, washed 3 times in PBS and incubated for 1 h with fluorescein isothiocyanate (FITC)-coupled goat anti-rabbit IgGs (BioTrend, Cologne, Germany), diluted 1:50 in PBS-BSA. After 3 rinses in PBS, cells were analysed in a Zeiss Photomicroscope with BP 485, FT 510, and LP 520 filters.

Miscellaneous

Protein concentration was determined using Bio-Rad protein assay reagents with BSA as a standard. Autoradiography was performed with silver-stained gels dried onto cellophane under vacuum and exposed to Amersham Hyperfilm-MP in Kodak X-Omatic cassettes with an intensifier screen for 3–48 h at –70°C. Phosphoproteins were evaluated densitometrically using a Quick Scan Densitometer from Desaga (Heidelberg, Germany). For quantitation, relative peak areas were measured.

RESULTS

We refined a cell fractionation protocol combining methods by Wright and Van Houten [31], Stelly et al [4] and Länge et al [8] to purify alveolar sacs. This minimises contamination by cilia, trichocysts, gullets, etc. of our preparations. Figure 1 shows the characteristic mosaic pattern of flattened membrane-bounded sacs [7]. Additionally, this protocol yielded an ER enriched fraction, as characterised by Wright and Van Houten [31].

As shown in Figure 2A, immunofluorescence analysis by SERCA-2A peptide specific ABs reveals a selective cortical staining in *Paramecium* cells. In Western blots of subcellular fractions, a strong immunoreactive band occurs at ~106 kDa either in isolated cortex fragments (containing different cortical structures, including alveolar

Fig. 2 Subcellular distribution of Ca^{2+} -ATPase in *P. tetraurelia* using SERCA-2A specific ABs (see also Discussion). (A) Immunofluorescence staining of fixed and permeabilised cells shows a selective cortical staining pattern. Magnification 700-fold. (B) Western blot analysis of subcellular fractions (50 μg) confirms strong immunoreactive band at ~106 kDa (arrowhead) either in isolated cortex-fragments (lane 2) or in alveolar sac fractions (lane 3), but none in plasma membrane vesicles (lane 4), microsomes (lane 5), trichocysts (lane 6) or in cilia (lane 7). Note lack of immunoreactivity in the 100,000 g pellet fraction (lane 1) due to insufficient amounts of cross-reactive protein.

sacs) or in alveolar sac fractions, but not in plasma membrane vesicles, microsomes, trichocysts or cilia (Fig. 2B), thus confirming subplasmalemmal localisation. Interestingly, the same immunoreactive band and the same fluorescent staining occur when the subcellular fractions were probed with ABs against a peptide from a recently cloned SERCA-type pump in *Paramecium* (see Figs 3, 4 in [36] and 'Materials and methods'). Feasibility is explained in the Discussion (see [37,38]).

Since all P-type Ca^{2+} -transport ATPases transiently form a characteristic Ca^{2+} -dependent phosphoenzyme

Fig. 3 EP formation in isolated alveolar sacs. Freshly prepared alveolar sacs were incubated for 20 s at 0°C with [γ - 32 P]-ATP with the following additions (as indicated below in figure): 0.1 mM CaCl₂, 0.3 mM EGTA (in lane 2 only), 1 mM MgCl₂, 50 μ M LaCl₃, 50 mM caffeine, 1% DMSO, 100 μ M TG, 100 μ M TGC, 100 μ M TBHQ, 100 μ M CPA. Acid-precipitated aliquots of alveolar sacs (8.5 μ g) were subjected to SDS-PAGE under Laemmli conditions and processed for autoradiography. (A) Silver-staining of a 5–10% SDS gel used for (B) EP formation autoradiography. Note that under standard conditions (lane 1) Ca²⁺-pump specific EP forms only at ~106 kDa (arrowhead) in a strong Ca²⁺- (lane 2) and Mg²⁺- (lane 3) dependent manner. La³⁺ can substitute for Mg²⁺ (lane 11) and stimulates EP formation (lane 10). EP formation is also increased by TG (lane 6) and TGC (lane 7) whereas TBHQ (lane 8) and CPA (lane 9) do not significantly influence EP formation. Also note that caffeine strongly inhibits EP formation (lane 4).

intermediate (EP) with hydroxylamine-labile acyl bonds [17], we analysed this aspect in freshly prepared alveolar sac fractions using well-defined assay conditions for mammalian Ca²⁺-ATPases of the SERCA-type [33]. In the presence of [γ - 32 P]-ATP, MgCl₂, and CaCl₂ phosphorylation can be visualised as 32 P-labelled 105–107 kDa

(average 106) protein in autoradiograms, not only on acidic gels (not shown), but also on Laemmli gels (Fig. 3, lane 1). Since this 32 P labelling is strongly sensitive to hydroxylamine (Fig. 4A) and also depends on the presence of Ca²⁺ and Mg²⁺ (Fig. 3, lanes 2 and 3) we assume the ~106 kDa protein to be the EP of the Ca²⁺-ATPase in alveolar sacs. Maximal labelling already occurred with 0.1 μ M Ca²⁺ (calculated and measured as in [3]), though we usually used higher concentrations as indicated in the figures. Also, the apparent molecular mass is more comparable to that of SERCAs [18,19] than to those of the plasma membrane. Also only the latter is calmodulin (CaM) regulated [17], just as in *Paramecium* where it forms EP at 133 kDa [31,39].

Lanthanum, an established inhibitor of Ca²⁺-transport ATPases, is generally used to discriminate between Ca²⁺-ATPases of the SERCA-type and those of the plasma membrane by either their inhibitory or stimulatory effects on EP formation, respectively [17]. Surprisingly, when pre-incubated in the presence of Ca²⁺ and Mg²⁺ (Fig. 3, lane 10) LaCl₃ (50 μ M) strongly increases EP-formation by a factor 2–3, an effect which seems to resemble that for the 133 kDa Ca²⁺-ATPase from *Paramecium* plasma membranes [31]. La³⁺ also increases EP formation in the presence of Ca²⁺ only (lane 11), which may indicate that it can substitute for Mg²⁺ (see also Table 1). In contrast, when Ca²⁺/Mg²⁺ was added after pre-incubation with La³⁺, EP formation is strongly reduced (Fig. 4B), thus showing characteristics similar to those observed with SERCA-type Ca²⁺-ATPases.

Also in Figure 3, we analysed the sensitivity of EP formation to either TG (TGC), TBHQ, or CPA which all are known to be specific, but structurally unrelated SERCA Ca²⁺-pump inhibitors [40]. In concentrations of 1 μ M which is sufficient to inhibit EP formation in mammalian SERCAs totally [41], 106 kDa EP formation is not influenced by any of these drugs, regardless of whether La³⁺ was absent or present and independently of the order of preincubations prior to the phosphorylation assay (not shown). However, on raising concentrations up to 100 μ M, phosphorylation intensity of the 106 kDa band does not decrease, but surprisingly increases by ~80% by either TG (Fig. 3, lane 6) or by the less hydrophilic analogue, TGC (lane 7). In contrast, TBHQ (lane 8) or CPA (lane 9) do not or only slightly influence EP formation even at high concentration. Since pre-incubation was in the presence of Ca²⁺ this may interfere with inhibition [42]. After pre-incubation prior to adding Ca²⁺ EP formation is increased in the same manner by TG or TGC, whereas no significant stimulation was observed with either TBHQ or CPA, respectively (not shown). We also analysed the influence of these inhibitors under conditions of intensified EP formation due to La³⁺. As shown in Table 1, EP formation was not significantly

Table 1 Densitometric evaluation of the influence of established SERCA inhibitors on EP formation of the Ca^{2+} -ATPase in subplasmalemmal Ca^{2+} -stores of *P. tetraurelia*

Assay conditions	Without La^{3+}	<i>n</i>	With La^{3+}	<i>n</i>
$\text{MgCl}_2/\text{CaCl}_2$	100	11	100	7
$\text{MgCl}_2/\text{EGTA}$	35.3 ± 6.3	11	37.5 ± 10.2	3
CaCl_2	11.9 ± 2.4	9	97.3 ± 17.0	3
$\text{MgCl}_2/\text{CaCl}_2/\text{DMSO}$	101.5 ± 13.5	7	78.3 ± 4.3	3
$\text{MgCl}_2/\text{CaCl}_2/\text{TG}$	181.6 ± 57.4	3	79.6 ± 6.4	3
$\text{MgCl}_2/\text{CaCl}_2/\text{TGC}$	174.7 ± 42.5	3	83.3 ± 1.7	3
$\text{MgCl}_2/\text{CaCl}_2/\text{t-BHQ}$	90.9 ± 18.4	4	111.6 ± 19.1	3
$\text{MgCl}_2/\text{CaCl}_2/\text{CPA}$	116.8 ± 21.2	5	121.6 ± 2.1	3

Alveolar sacs were assayed in the presence or absence of $50 \mu\text{M}$ LaCl_3 , as in Figure 3, with the following additions: 1 mM MgCl_2 , 0.1 mM CaCl_2 , 0.3 mM EGTA, 1% DMSO, $100 \mu\text{M}$ TG, $100 \mu\text{M}$ TGC, $100 \mu\text{M}$ TBHQ, $100 \mu\text{M}$ CPA. Aliquots of $8.5 \mu\text{g}$ were subjected to SDS-PAGE and processed for autoradiography. *n* = number of experiments, $\pm$ = SEM.

Fig. 4 Influence of hydroxylamine (A) or of La^{3+} pre-incubation (B) on $\sim 106 \text{ kDa}$ EP formation. Autoradiograms of SDS-PAGE separated alveolar sacs ($16 \mu\text{g}/\text{lane}$) assayed in the presence of $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ under following conditions. (A) Hydroxylamine (HA) sensitivity of EP formation was tested as described in Materials and methods. Formation of EP at $\sim 106 \text{ kDa}$ (arrowhead) is sensitive to 150 mM HA (lane 2) compared to the control (lane 1) without HA. (B) In lane 2, Ca^{2+} (0.1 mM) and Mg^{2+} (1 mM) was added to the reaction mixture prior to LaCl_3 ($50 \mu\text{M}$). In lane 4, $\text{Ca}^{2+}/\text{Mg}^{2+}$ was added after pre-incubation with LaCl_3 . Depending on the order of pre-incubation, La^{3+} either increases (lane 2) or inhibits (lane 4) EP formation at $\sim 106 \text{ kDa}$ (arrowhead) when compared to controls, $-\text{La}^{3+}$ (lanes 1 and 3).

influenced by any inhibitor. Therefore, La^{3+} may act on similar sites of the Ca^{2+} -ATPase molecule as TG or TGC in high concentrations. Altogether, these data would not yet unequivocally identify the $\sim 106 \text{ kDa}$ protein as a SERCA-type, but one has to consider the well-known insensitivity of a variety of Ca^{2+} stores to different inhibitors [43].

Hence, we also tested the effect of added endogenous CaM on EP formation. As shown in Figure 5, CaM does not stimulate EP formation (lane 3). Since this might be attributed to CaM present in alveolar sac fractions (for which we have evidence from Western-blots, not shown), we also tested the effects of CaM inhibitors. However,

Fig. 5 Lack of influence of CaM and CaM antagonists on EP formation at $\sim 106 \text{ kDa}$. Autoradiogram of SDS-PAGE separated alveolar sacs ($18 \mu\text{g}/\text{lane}$) analysed after specific $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ labelling under standard conditions (lane 1), either in the presence of $2 \mu\text{M}$ *Paramecium* CaM (lane 3), $20 \mu\text{M}$ R24571 (lane 5) or $100 \mu\text{M}$ TFP (lane 6). Controls were also run in the presence of 1% DMSO in which R24571 had been dissolved (lane 4) or in the presence of 0.5 mM EGTA to demonstrate Ca^{2+} -dependency (lane 2). Note that CaM (lane 3) and TFP (lane 6) do not change EP formation in the presence of 1 mM MgCl_2 and 0.1 mM CaCl_2 (lane 1). The same is true for R24571 (lane 5), when compared to the effects of DMSO which slightly increases EP formation at $\sim 106 \text{ kDa}$ (lane 4).

calmidazolium and TFP (lanes 5 and 6) do not influence EP formation. To check any possible loss of a putative CaM-binding domain, more protease inhibitors were used during isolation at higher concentration. However, neither an EP shift to higher apparent molecular masses, nor any effects on phosphorylation are observed in the presence of CaM or of CaM antagonists (not shown). This strongly supports CaM-independency of the $\sim 106 \text{ kDa}$ EP, thus arguing for a SERCA-type.

We also checked the influence of orthovanadate, a stereo-analogue of phosphate which at low concentrations strongly inhibits the activity of CaM-regulated Ca^{2+} -ATPases by binding to the amino acid residue

Fig. 6 Influence of vanadate and caffeine on ~106 kDa EP formation. For autophosphorylation reaction, alveolar sacs (15 μg/lane) were analysed in the presence of 3 nM [γ -³²P]-ATP (4500 Ci/mmol), 1 mM MgCl₂, 0.1 mM CaCl₂ and either increasing caffeine (≤ 100 mM, lanes 1–6) or 30 μM vanadate (lane 8). Compared to controls, in the presence of Ca²⁺ and Mg²⁺ (lane 7), caffeine strongly inhibits EP formation at ~106 kDa (arrowhead) in a dose-dependent manner, whereas vanadate does not significantly inhibit autophosphorylation.

relevant for EP formation at the active site [17]. When tested up to 30 μM, i.e. 5–15-fold higher than observed for half-maximal inhibition of the Ca²⁺-ATPase in *Paramecium* plasma membranes [30,31], EP-formation is not or only slightly inhibited, maximally by ~25% (Fig. 6, lane 8). This is comparable to effects on EP formation [44] or calcium uptake with established SERCAs [45]. Ruthenium red, a polycationic dye which non-specifically inhibits the plasma membrane Ca²⁺-pump [46] by interacting with inositide-dependent sites on the molecule [47] also does not influence EP formation in the alveolar sacs (≤ 30 μM tested, not shown).

We then analysed whether caffeine, an activator of Ca²⁺ release channels of intracellular stores in other systems [13–15], may have any effect on the Ca²⁺-ATPase of alveolar sacs, as one might conclude from ⁴⁵Ca²⁺ uptake kinetics [10]. Again we analysed EP formation under varying conditions (Figs 6–8). In fact, caffeine strongly inhibits EP formation in a dose dependent manner (Fig. 6), with IC₅₀ = 10 mM when pre-incubated in the presence of Ca²⁺ and Mg²⁺ (Fig. 8). A similar inhibition is observed, when caffeine effects were

Fig. 7 Influence of caffeine on the EP formation in alveolar sacs and skeletal muscle SR. (A) Silver-stained proteins (5 μg) of alveolar sacs (lanes 1–6) and of SR (lanes 7–12) on a 5–10% SDS gel used for autoradiography in (B). Autophosphorylation assays by [γ -³²P]-ATP were performed in the presence of 1 mM MgCl₂, 0.1 mM CaCl₂, 50 μM LaCl₃ and increasing concentrations of caffeine. For visualising EP formation, autoradiograms were exposed 3 h (SR) or 2 days (alveolar sacs). Note that EP formation in alveolar sacs at ~106 kDa (small arrowhead) is inhibited by caffeine in a dose-dependent manner (lanes 2–6), in contrast to the ~110 kDa of the SR Ca²⁺-pump (large arrowhead, lanes 8–12).

analysed under conditions of intensified EP formation due to 50 μM La³⁺ (Fig. 7, lanes 1–6), with IC₅₀ = 9 mM (Fig. 8). When assayed under conditions of reduced EP formation (Ca²⁺/Mg²⁺ added after La³⁺), IC₅₀ = 50 mM. In contrast, with Ca²⁺/Mg²⁺ added after caffeine, in the absence of La³⁺, EP formation is increased by 50 mM caffeine by ≤ 40% (Fig. 8, filled circles).

Altogether, these data strongly suggest that caffeine may directly act on the SERCA, i.e. under normal conditions by inhibition. For concentration dependent caffeine effects, see also end of Discussion. To check whether caffeine would inhibit other SERCAs as well, we analysed microsomes from fast-twitch fibres of rabbit skeletal muscle containing SERCA-1 [18,32]. EP formation at ~110 kDa shows strong TG/TGC sensitivity, also under such conditions when EP formation in *Paramecium* alveolar sacs has been stimulated (not

Fig. 8 Inhibition of EP formation in alveolar sacs by caffeine depends on assay conditions. Relative peak areas from autoradiograms were evaluated from autophosphorylations in alveolar sacs (15 μ g/lane), assayed as in Figures 6 and 7, where pre-incubation with MgCl_2 (1 mM) and CaCl_2 (0.1 mM) was in the presence (stars) or absence (filled squares) of 50 μ M LaCl_3 prior to addition of increasing caffeine concentrations. Alternatively, assays were run when MgCl_2 and CaCl_2 were added after pre-incubation with caffeine (filled circles) or caffeine plus 50 μ M LaCl_3 (filled diamonds). Note that under conditions such as in Figures 6 and 7, caffeine strongly inhibits EP formation with or without LaCl_3 with $\text{IC}_{50} = 9$ mM (stars) or 10 mM (filled squares), respectively. When $\text{Ca}^{2+}/\text{Mg}^{2+}$ was added after pre-incubation with caffeine plus 50 μ M La^{3+} , half-maximal inhibition occurs at ~ 50 mM for caffeine (filled diamonds), whereas a slight stimulation is observed in the absence of La^{3+} (filled circles).

shown). When tested for caffeine sensitivity under varying conditions, EP formation was not significantly inhibited by ≥ 50 mM caffeine, independently of whether La^{3+} was present (Fig. 7, lanes 7–12) or not (not shown).

DISCUSSION

Although there are several reports on active calcium sequestration into the alveolar system of *Paramecium* cells [4,8,10], the unequivocal identification and biochemical characterisation of a Ca^{2+} -ATPase occurring in the membranes of these subplasmalemmal calcium stores has not been accomplished. We now present further data on the existence of such a Ca^{2+} -ATPase located exclusively in these stores which shares several immunochemical, enzymatic, pharmacological and biochemical characteristics with known mammalian Ca^{2+} -ATPases of the SERCA-type. However, there are also some unusual features, which will be discussed in detail.

Localisation in subplasmalemmal stores

Alveolar sacs in the cortex of *Paramecium* cells constitute a subplasmalemmal Ca^{2+} storage compartment [4–6,8–10,

48]. By combining several isolation protocols [4,8,31] we obtained an alveolar sac fraction predominantly free of cilia, trichocysts, gullets and other contamination (Fig. 1). This is supported by identification in these stores of a Ca^{2+} -ATPase which we consider related to SERCA-type pumps for the following reasons:

1. SERCA specific ABs only react with alveolar sacs or with isolated cortex fragments (containing alveolar system), but not with isolated plasma membrane vesicles, cilia, microsomes, or trichocysts (Fig. 2B). This localisation is confirmed by the exclusive cortical localisation of immunofluorescence in permeabilised cells (Fig. 2A).
2. The size of the immunoreactive band on Western blots and localisation of immunofluorescence is identical as with ABs against a peptide, 506-SSQNEKGNVLFIKG-519, from the cloned SERCA-type Ca^{2+} pump of the same calcium stores of *Paramecium* (see Figs 3 & 4 in [36]).
3. The gene product from *Paramecium* contains the sequence, 966-YLXXAI-972 [36], which is also found in the C-terminal region of SERCA-2A [35]. Epitopes of this type can be recognised by ABs [37,38].
4. The molecular mass of ~ 106 kDa, obtained either by Western blot analysis (Fig. 2B) or by EP formation (Figs 3–7), strongly resembles SERCA-type Ca^{2+} -ATPases [18], rather than *Paramecium* Ca^{2+} -ATPases of 146 kDa in the ciliary membrane [49], of 53–68 kDa in the ciliary supernatant fraction [50], or at 131–133 kDa in the somatic plasma membrane [39,51], for which we confirm EP formation (not shown).
5. Since formation of hydroxylamine-labile phosphoenzyme intermediates in alveolar sacs strongly depends on Ca^{2+} and Mg^{2+} , but not on CaM, CaM antagonists, vanadate or ruthenium red, this also excludes plasma membranes as source of EP formation [30,31,39].
6. All this is corroborated by identification of a cDNA encoding a *Paramecium* SERCA gene [36] which is quite different from the recently cloned plasmalemmal Ca^{2+} -pump [51].

Assignment to the SERCA-type

Can our enzymatic data, including some unorthodox characteristics (such as the effects of La^{3+} , low sensitivity to SERCA inhibitors, inhibition by caffeine) be reconciled with assignment to a SERCA type? A most important criterion is hydroxylamine-sensitivity of the phosphoenzyme intermediates (Fig. 4A) and its formation in a

Ca^{2+} - and Mg^{2+} -dependent manner (Fig. 3) at 0°C with fast kinetics (20 s), thus distinguishing them from potential kinase substrates [33]. Since this has been observed with the alveolar sac fractions (Fig. 3) with known $^{45}\text{Ca}^{2+}$ sequestration activity [4,8,10] this accords with a similar reaction mechanism accepted for all Ca^{2+} sequestering ATPases analysed so far [17,18,52,53]. When ATP is added after pre-incubation with Ca^{2+} , EP may be rapidly formed by transfer of the ATP terminal phosphate (E_1P) to a hydroxylamine-sensitive aspartyl residue, in *Paramecium* probably at position 360 [36]. In parallel with EP formation, before hydrolytic cleavage of P_i , bound Ca^{2+} may be internalised by the enzyme and then released into the vesicle lumen against a concentration gradient. In the resting state the total Ca^{2+} concentration has been estimated to be in the range of that in SR [6]. This transition to the E_2P conformation may be promoted by Mg^{2+} . The catalytic and transport cycle would then be finally completed by hydrolytic cleavage of the phosphorylated enzyme intermediate (E_2) followed by a return to the high-affinity conformation E_1 state. However, such a reaction mechanism is not restricted to Ca^{2+} -ATPases of the SERCA type, but is also applied to plasmalemmal Ca^{2+} -ATPases [17,19]. Therefore, other criteria are required to distinguish between these two types of Ca^{2+} -ATPases [17,41]. One of these is La^{3+} which, at variance with the SERCA Ca^{2+} -pump, at low concentrations ($\leq 100 \mu\text{M}$), increases the steady-state level of the phosphoenzyme in the plasmalemmal Ca^{2+} -pump [17]. Unexpectedly, with the Ca^{2+} -ATPase in alveolar sacs, we observed both stimulatory and inhibitory effects of La^{3+} on EP formation depending on the sequence of application relative to $\text{Ca}^{2+}/\text{Mg}^{2+}$ prior to the phosphorylation reaction (Fig. 4B). These antagonistic effects of La^{3+} may be explained either by the displacement of Mg^{2+} (interrupting the $\text{E}_1\text{P} \rightarrow \text{E}_2\text{P}$ transition, with accumulation of E_1P , as is discussed for the plasmalemmal Ca^{2+} -ATPase [54] and for SERCAs [55,56]), or by direct binding to cytoplasmic Ca^{2+} transporting sites (blocking phosphorylation with the consequent low level, as observed with SERCAs [57]). However, since we found the effects of La^{3+} to depend strongly on the sequence of pre-incubation prior to the reaction, such a criterion cannot definitely discriminate between the two types of Ca^{2+} -ATPases.

Another potential criterion is the use of SERCA specific inhibitors. TG at nanomolar concentrations [58] is believed to bind stoichiometrically to all SERCAs and causes irreversible inhibition of their activity by blocking in the Ca^{2+} -free E_2 state [59]. However, when tested at concentrations of $1 \mu\text{M}$, EP formation in alveolar sacs is not influenced (this paper) and $^{45}\text{Ca}^{2+}$ -pumping activity is not reduced [8,10]. The same is true for conditions (TG applied prior to Ca^{2+}) when a protective effect of Ca^{2+} on

the E_1 conformation [60] can be excluded. Surprisingly, in concentrations up to $100 \mu\text{M}$, the EP states (either E_1P or E_2P) are preferred (Fig. 3) suggesting that TG or TGC may also exhibit some other inhibitory effects on the *Paramecium* enzyme which, however, have still to be analysed in detail. Similarly, at these concentrations, $^{45}\text{Ca}^{2+}$ sequestration is reduced [8,10]. We conclude the SERCA of alveolar sacs to be of low TG sensitivity. TG-binding domains in the pump we analysed are at variance to those in TG-sensitive stores [36]. Occurrence of low TG-sensitivity organellar Ca^{2+} -ATPases has also been demonstrated in bovine adrenal chromaffin cells [61] or in human platelets [56,62], the latter also exhibiting an increase of steady-state EP level in the presence of La^{3+} . Ca^{2+} -ATPases of low TG sensitivity also occur in Golgi fractions from yeast [63], in the ER of several plants [64,65], as well as in Ca^{2+} -storage compartments of the protozoan parasite *Leishmania mexicana* [66]. In several mammalian cell lines, co-existence of TG-sensitive and insensitive pools has been postulated [67–69].

Effects of caffeine

Quite unexpected was inhibition of EP formation by caffeine (Figs 6–8). Interestingly, with regard to the situation in *Paramecium*, in GH_4C_1 cells a TG-resistant, ATP-dependent Ca^{2+} -storage seems to be coupled to the caffeine-responsive pool with characteristics of a CICR compartment [67]. However, in *Paramecium* there is no evidence for such a release mechanism [8,16], although caffeine clearly mobilises cortical Ca^{2+} in vivo very rapidly, i.e. within 2 s [11]. On the other hand, $^{45}\text{Ca}^{2+}$ uptake kinetics with alveolar sac fractions revealed non-competitive inhibition [10]. This is in good agreement with the present work on EP formation (Figs 6–8). Thus, caffeine may exert a dual effect in the protozoan system, i.e. rapid store mobilization [11] and inhibition of Ca^{2+} sequestration into stores (this study). Since the effects of caffeine also depend on assay conditions (see Results and Fig. 8) we assume that caffeine may interfere with the actions of Mg^{2+} on the various conformations of the enzyme which can promote both EP composition and decomposition [17]. Although caffeine is required in similarly high concentrations, i.e. $\geq 50 \text{ mM}$, for full Ca^{2+} response also in higher eukaryotic systems [70,71], it remains to be seen whether the caffeine effects we describe are applicable also to other systems.

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