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Ca^{2+} sequestering in the early-branching amitochondriate protozoan *Tritrichomonas foetus*: an important role of the Golgi complex and its Ca^{2+}-ATPase

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Abstract

Total membrane vesicles isolated from *Tritrichomonas foetus* showed an ATP-dependent Ca^{2+} uptake, which was not sensitive to 10 μM protonophore FCCP but was blocked by orthovanadate, the inhibitor of P-type ATPases (I_{50} = 130 μM), and by the Ca^{2+}/H^+ exchanger, A-23187. The Ca^{2+} uptake was prevented also by thapsigargin, an inhibitor of the SERCA Ca^{2+}-ATPases. The sensitivity of the Ca^{2+} uptake by the protozoan membrane vesicles to thapsigargin was similar to that of Ca^{2+}-ATPase from rabbit muscle sarcoplasmic reticulum. Fractionation of the total membrane vesicles in sucrose density gradient revealed a considerable peak of Ca^{2+} transport activity that co-migrated with the Golgi marker guanosine diphosphatase (GDPase). Electron microscopy confirmed that membrane fractions of the peak were enriched with the Golgi membranes. The Golgi Ca^{2+}-ATPase contributed to the Ca^{2+} uptake by all membrane vesicles 80–85%. We conclude that: (i) the Golgi and/or Golgi-like vesicles form the main Ca^{2+} store compartment in *T. foetus*; (ii) Ca^{2+} ATPase is responsible for the Ca^{2+} sequestering in this protozoan, while Ca^{2+}/H^+ antiporter is not involved in the process; (iii) the Golgi pump of this ancient eukaryotic microorganism appears to be similar to the enzymes of the SERCA family by its sensitivity to thapsigargin.

Keywords: *Tritrichomonas foetus*; Ca^{2+} homeostasis; Golgi complex; Ca^{2+}-ATPase

1. Introduction

Ca^{2+} is the principal regulatory ion and therefore different transporters which are involved in its homeostasis in various organisms from bacteria to man are under intensive investigation [1]. It is currently accepted that the sarcoplasmic/endoplasmic reticulum (ER) plays a main role in Ca^{2+} storage in mammalian cells, while a vacuole is the principal store compartment in plants, fungi and yeast [1–4]. There is, however, the evidence that all organelles of the yeast secretory pathway are involved in Ca^{2+} homeostasis and are equipped with different Ca^{2+}-ATPases and Ca^{2+}/H^+ antiporter(s) [5,6].

Ca^{2+} homeostasis and signaling in the parasitic protozoa and especially in *Trypanosoma cruzi* and *Trypanosoma brucei* is of special interest, since they cause Chagas disease and sleeping sickness, respectively [7]. Genes for Ca^{2+}-ATPases of intracellular membranes of *T. brucei* and *Leishmania mexicana amazonensis* were cloned [8,9] and the presence of Ca^{2+}-ATPase in the plasma membrane or membranes which co-migrated with those ones of *T. brucei* and *T. cruzi* was demonstrated [10]. Furthermore, the existence of a special organelle, the acidocalcisome, for Ca^{2+} storage in *T. cruzi* and other protozoa has been

Abbreviations: DMSO, dimethyl sulfoxide; DTT, dithiothreitol; ER, endoplasmic reticulum; FCCP, carbonyl-cyanide *p*-trifluoromethoxyphenylhydrazone; ΔpH, a chemical H^+ gradient; PMSF, phenylmethylsulfonyl fluoride; POPOP, (1,4-bis[5-phenyl-2-oxazolyl]-benzene; 2,2′-*p*-phenylene-bis[5-phenyloxazole]); PPO, 2,5-diphenyloxazole; SERCA, sarcoplasmic endoplasmic reticulum calcium ATPases

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suggested [11,12]. It is rather unfortunate that little is known about the Ca^{2+} homeostasis in such members of trichomonads as *Tritrichomonas foetus*.

T. foetus is a parasitic protozoan from the urogenital tract of cattle which causes abortion and sterility. This protozoan, among other parasites like *Giardia* and *Entamoeba*, is considered to be an ancient microorganism, placed to the basis of Eukarya evolutionary tree [13,14]. It lacks mitochondria, but possesses the double-membrane-bound organelles, hydrogenosomes, that produce molecular hydrogen and ATP [15,16]. Moreover, the results of electron microscopic analysis suggest that hydrogenosomes could be also involved in Ca^{2+} sequestering [17].

It is widely accepted that a main role in Ca^{2+} sequestering in mammalian cells is carried out by the ER and its Ca^{2+}-ATPases, which are encoded by SERCA 1–3 genes [1,18]. Vacuoles are believed to be the main Ca^{2+} store compartments in plants, fungi and yeast [2–4,19–22]. In this case V H^+-ATPase creates an electrochemical H^+ gradient across tonoplast and a chemical component (ΔpH) is used by Ca^{2+}/H^+ antiporter for the Ca^{2+} accumulation inside of vacuoles [21,22]. The activity of this antiporter is blocked by protonophores, which collapse the H^+ gradients, while Ca^{2+}-ATPases are inhibited in presence of orthovanadate, which has no effect on the V H^+-ATPase and the vacuolar Ca^{2+}/H^+ antiporter [1–3,5,6,23].

The aim of the present work has been to identify Ca^{2+} sequestering organelles and their Ca^{2+} transporters in *T. foetus*, as well as to evaluate their contribution to the Ca^{2+} storage by the whole protozoan cell.

2. Materials and methods

2.1. Cell culture

We used the K strain of *T. foetus* which was isolated by Dr. H. Guida (EMBRAPA, Rio de Janeiro, Brazil) from the urogenital tract of a bull. It was maintained in TYM Diamond Medium [25] and cultivated at 37 °C for 24 h, which corresponds to the end of the logarithmic phase of the growth.

2.2. Reagents

ATP, DMSO, sodium orthovanadate and toluene were from Merck (Darmstadt, Germany). Thapsigargin was from Molecular Probes Inc. (OR, USA) and other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.3. Membrane fractionation

Cells were washed in the homogenization buffer (20 mM HEPES buffer, pH 7.2, 365 mM sucrose, 1 mM $MgSO_4$ and 1 mM DTT) and then disrupted in it in presence of benzamidine (1 mM), PMSF (1 mM) and of the cocktail of the polypeptide protease inhibitors containing antipain, aprotinin, chymostatin, leupeptin and pepstatin, each one at final concentration of 1 μg/ml [6]. Cells were disrupted in Dounce homogenizer at 0–4 °C. The homogenate was then centrifuged at 4° for 10 min at $1500 \times g$ and total membranes were obtained from the resulting supernatant by centrifugation at $45{,}000 \times g$ for 40 min. The total membranes were resuspended in the homogenization buffer in the presence of protease inhibitors and used either for further analysis or layered onto a sucrose gradient. The gradient was made of 10 steps of sucrose concentration from 63% to 30% (w/w), according to Ref. [6]. After 2 h 45 min of centrifugation at $130{,}000 \times g$, membrane fractions were collected using RediFrac sample collector and P1 peristaltic pump (Pharmacia-Biotech) and immediately frozen.

2.4. $^{45}Ca^{2+}$ uptake

$^{45}Ca^{2+}$ uptake was determined by the filtration technique as indicated in Refs. [6,24], using 0.45-μm nitrocellulose filters HAWP (Millipore, USA) and the H. Hölcel filtration apparatus (Dorfer, Germany). ATP was omitted in control experiments to determine the ATP-independent $^{45}Ca^{2+}$ binding. Ca^{2+}-free concentration of 1 μM was maintained with EGTA according to Ref. [26]. Oxalate, 20 mM, was used in assays of the $^{45}Ca^{2+}$ uptake inhibition by thapsigargin. Radioactivity was measured with a toluene–PPO–POPOP cocktail using the Packard scintillation counter, model 1600TR.

2.5. Inhibitor analysis

To determine an effect of different inhibitors on Ca^{2+} uptake by total membrane vesicles or by membrane fractions isolated from *T. foetus*, we used 1–10 μM of the protonophore FCCP to collapse the H^+ gradient across the membranes and, therefore, to block the activity of Ca^{2+}/H^+ antiporter(s). To distinguish Ca^{2+}-ATPases, we used the inhibitor of P-type ATPases, sodium orthovanadate (90–1000 μM), and the inhibitor of the SERCA, thapsigargin (1–6.25 μM). In the case of thapsigargin assays, membrane vesicles were preincubated with the inhibitor for 10 min on ice. In the case of FCCP and orthovanadate assays the preincubation with an inhibitor was omitted. The solutions of FCCP and A-23187 were prepared in ethanol and thapsigargin was dissolved in DMSO. The calcium uptake conditions were the same as described above and the corresponding controls for each concentration of DMSO or ethanol were included.

2.6. Marker enzyme activities

Activities of the Golgi marker enzyme guanosine diphosphatase (GDPase) and the hydrogenosome marker malic enzyme, L-malate:NADP oxidoreductase, were determined

according to published protocols [27,28]. Protein content was measured according to Ref. [29]. The activities were determined using Shimadzu spectrophotometers, models UV-1203 and UV-160A.

2.7. Electron microscopy

Cells were washed twice in phosphate buffered saline (PBS), pH 7.2 and fixed in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 h at room temperature. After fixation, samples were washed in 0.1 M cacodylate buffer and post-fixed for 15 min in 1% (w/v) OsO_4 in 0.1 M cacodylate buffer plus 0.8% potassium ferricyanide. Selected membrane fractions separated on sucrose density gradient were fixed for 30 min at room temperature in the same glutaraldehide/cacodilate solution as whole cells. Membrane fractions or whole cells were then dehydrated in crescent series of acetone and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and observed in Zeiss 900 electron microscope.

3. Results

3.1. Ca^{2+}-ATPase is the principal contributor to the Ca^{2+} sequestering in T. foetus

Total membranes isolated from *T. foetus* cells showed a time-dependent and ATP-stimulated Ca^{2+} uptake (Fig. 1). Accumulated Ca^{2+} was retained by membrane vesicles for at least 30 min, indicating that they were stable during the experiment. A similar characteristic of the Ca^{2+} uptake was also found when the uptake was measured in the presence of 20 mM oxalate. The latter forms insoluble complexes with Ca^{2+} in the lumen of the membrane vesicles and facilitates its sequestering. By doing so, the steady state of Ca^{2+} accumulation was increased up to 20–30 times (not shown). This indicates the high capacity of the *T. foetus* membrane vesicles to sequester Ca^{2+} when the appropriate Ca^{2+} binding molecules are available in the lumen.

In order to determine either Ca^{2+}-ATPases or Ca^{2+}/H^+ antiporters or both types of Ca^{2+} transporters operate in membrane vesicles of *T. foetus*, we analyzed the effect of protonophore FCCP and some inhibitors of Ca^{2+}-ATPases on Ca^{2+} uptake. The Ca^{2+}/H^+ antiporter can work only when a difference of the chemical potentials of H^+, ΔpH, is supplied [20–22].

Notably, FCCP at concentration of 1 μM did not inhibit Ca^{2+} uptake (Fig. 1). Similar results showing independence of Ca^{2+} uptake from Δ pH were obtained with 10 μM FCCP both in the presence of oxalate and in its absence (not shown). In agreement with these data, bafilomycin A_1, a potent inhibitor of V H^+-ATPases, did not inhibit Ca^{2+} transport by total membrane vesicles (not shown). We therefore conclude that the Ca^{2+} transport by vesicles of the *T. foetus* total membranes is not driven by ΔpH and that Ca^{2+}/H^+ antiporter does not contribute to the Ca^{2+} uptake.

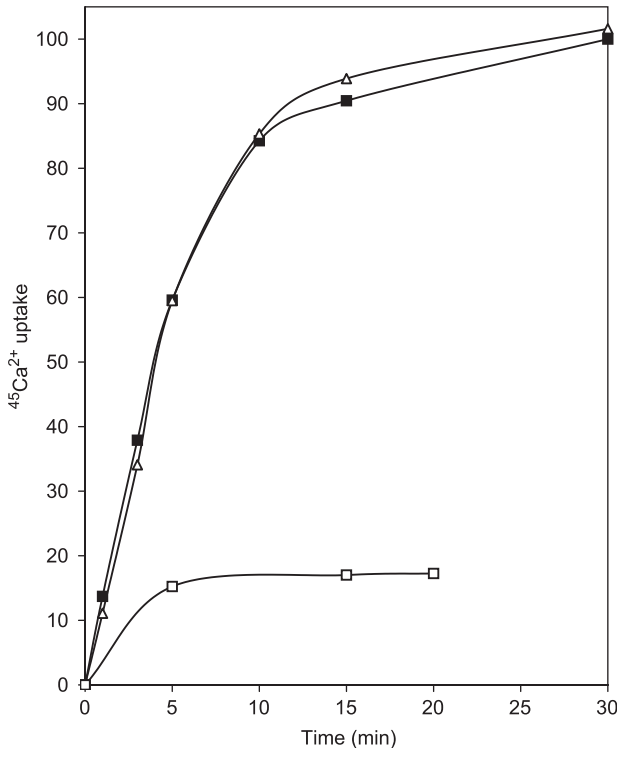

Fig. 1. Time- and ATP-dependent $^{45}Ca^{2+}$ uptake by vesicles of total membranes of *T. foetus*. The incubation medium of 1.5 ml contained 150 μg of protein. Samples of 180 μl were taken at time indicated, filtered and washed (details are in Materials and methods). The medium contained ATP (■) (1 mM), ATP and 1 μM FCCP (△) or no ATP (□). One-hundred percent of the Ca^{2+} uptake corresponds to 2.4 nmol Ca^{2+}/mg protein × 10 min^{-1}.

The ionophore A-23187, which mediates $Ca^{2+}/2H^+$ exchange, significantly prevented Ca^{2+} uptake at concentration 5 μM when added before ATP (not shown). It completely released the pre-accumulated Ca^{2+} when added after 10 min of Ca^{2+} uptake (Fig. 2). The latter data can be taken as an indication that Ca^{2+} was transported into membrane vesicles against its concentration gradient. It is noteworthy that Ca^{2+} uptake was blocked by orthovanadate, an inhibitor of the P-type ion transporting ATPases including Ca^{2+}-ATPases (Fig. 3). The half inhibition of Ca^{2+} uptake was found with 130 μM of this inhibitor. This concentration of orthovanadate is more likely to inhibit the intracellular membrane Ca^{2+}-ATPases than the plasma membrane Ca^{2+}-ATPases, which show higher sensitivity to orthovanadate [1,9]. Moreover, thapsigargin, a classical inhibitor of the SERCA [18], blocked 80% of Ca^{2+} uptake by total membrane vesicles of *T. foetus* at concentration 3 μM (Fig. 4). Since this concentration of the inhibitor is higher than the one which can completely block activity of the Ca^{2+} pumps of the SERCA family (100 nM, [18,30]),

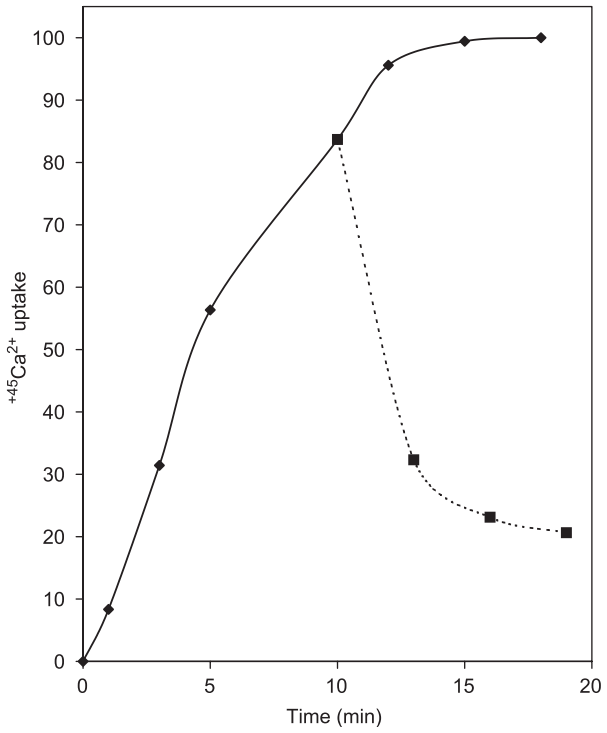

Fig. 2. Ionophore A-23187 released pre-accumulated Ca^{2+} in total membrane vesicles of *T. foetus*. Total membrane vesicles were separated in two parts; the first part received DMSO after 10 min of Ca^{2+} uptake, while 5 μM A-23187 was simultaneously added to the second one. One-hundred percent of the sequestered Ca^{2+} corresponds to 3.0 nmol Ca^{2+}/mg protein × 10 min^{-1}.

we tested the inhibitor with the classical target enzyme from rabbit muscles. It is clear from Fig. 4 that the enzymes of rabbit sarcoplasmic reticulum and of *T. foetus* show similar sensitivity to thapsigargin. Taking into account the ratio of the inhibitor to protein content in the assay system, one can see that 50% of inhibition of Ca^{2+} uptake by membranes from sarcoplasmic reticulum and *T. foetus* occurred at 11.5 and 17 nmol/mg, respectively (Fig. 4). Thus, the Ca^{2+}-ATPase of the total membranes of *T. foetus* does not differ essentially from Ca^{2+}pump of rabbit muscles by its sensitivity to thapsigargin and orthovanadate [18,30]. However, the enzyme from *T. foetus* is slightly more resistant to thapsigargin.

Overall, as a result of the inhibitory analysis, Ca^{2+} uptake by total membrane vesicles isolated from *T. foetus* is mediated by a primary transporter, that is Ca^{2+}-ATPase, and Ca^{2+}/H^{+} antiporter does not contribute to the Ca^{2+} sequestering in this protozoan.

3.2. The Golgi apparatus is the main Ca^{2+} store compartment of T. foetus

To identify the organelles involved in Ca^{2+} uptake by total membrane vesicles and therefore in Ca^{2+} homeostasis in *T. foetus*, total membranes were fractionated on a sucrose density gradient. Ca^{2+} uptake and marker enzyme activities were then determined in each membrane fraction. Fig. 5 shows that ATP-dependent Ca^{2+} transport activity was found in several populations of membrane vesicles that migrated at different sucrose densities. The Ca^{2+} uptake by any membrane fraction (Fig. 5) was not sensitive to FCCP (not shown), suggesting again a key contribution of Ca^{2+}-ATPase(s) in the formation and refilling of Ca^{2+} store pools in this protozoan. A major peak of Ca^{2+} transport activity was detected at 35% sucrose where one can expect a migration of the Golgi and/or Golgi-like membrane vesicles (Fig. 5a). The determination of GDPase, a marker enzyme of Golgi [27], revealed that its activity migrated exactly in the same region of the gradient (Fig. 5a, fractions 31–41). These membrane vesicles contributed up to 85% of the Ca^{2+} uptake activity and up to 70% to the GDPase activity of all fractions.

Membrane vesicles derived from organelles different from the Golgi also contributed to the Ca^{2+} uptake. This was clear when the Ca^{2+} uptake activity in membrane fractions 32–45 was decreased 15-fold in the comparison with the activity of fractions 1–31 (Fig. 5b). The determination of malic enzyme activity, the marker of hydrogenosomes [28], revealed that it was found at 51% of the sucrose gradient in the region of heavy membrane vesicles (Fig. 5a, fractions 12–22) where one can also detect the plasma membrane vesicles and rough ER vesicles [6]. The contri-

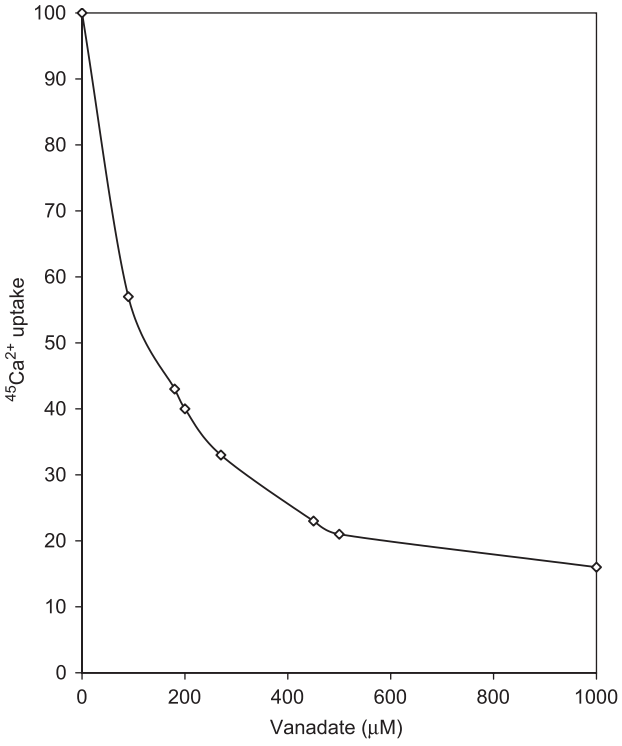

Fig. 3. Orthovanadate inhibits $^{45}Ca^{2+}$ uptake by total membrane vesicles of *T. foetus*. $^{45}Ca^{2+}$ uptake stimulated by ATP in the absence of orthovanadate is taken as 100% which equals to 2.8 nmol/mg protein × 10 min^{-1}.

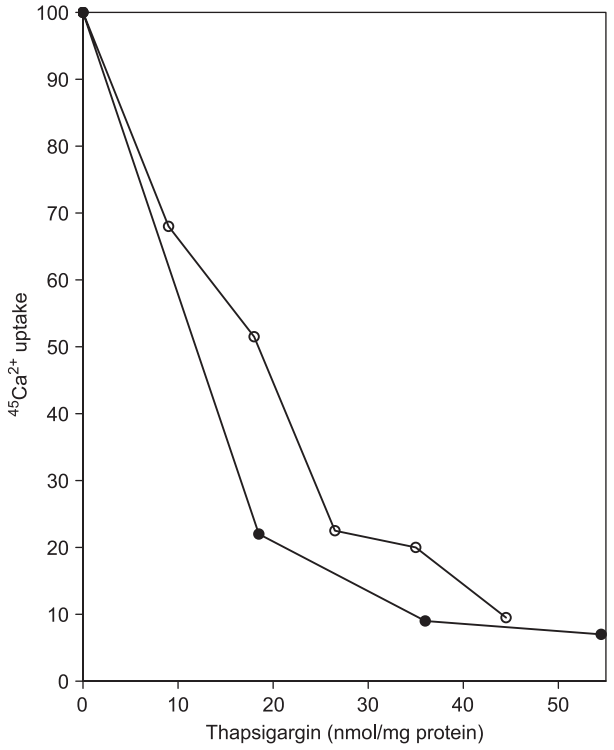

Fig. 4. Thapsigargin inhibits $^{45}Ca^{2+}$ uptake by total membrane vesicles of *T. foetus* (○) and by vesicles of the rabbit sarcoplasmic reticulum (●). Oxalate 20 mM was added to the incubation medium. One-hundred percent of the Ca^{2+} uptake corresponds to 67 nmol/mg protein × 10 min^{-1}. Final concentrations of thapsigargin were 1, 2, 3, 4 and 5 μM for membranes of *T. foetus* and 1, 2 and 3 μM for sarcoplasmic reticulum.

bution of the membrane fractions 13–22 in the total activity of malic enzyme reached 47%, whereas their contribution to Ca^{2+} transport activity was not higher than 4.5%. However, Ca^{2+} transport activity of heavy membrane vesicles partly co-migrated with the malic enzyme activity (Fig. 5), suggesting a possible minor contribution of hydrogenosomes to the ATP-dependent Ca^{2+} accumulation and, therefore, to Ca^{2+} storage.

We further characterized some membrane fractions by an electron microscopic analysis. Fig. 6a shows a general view of the protozoan as seen in thin section. Such typical structures as the hydrogenosomes, the Golgi complex, nucleus and glycogen granules can be easily identified. There were only a few profiles of the endoplasmic reticulum (Fig. 6a, arrows), in contrast to the typical Golgi complex (Fig. 6b). The electron microscopic analysis of the membrane fractions 14–19, enriched with malic enzyme activity, revealed a large number of hydrogenosomes, although many glycogen granules were also observed (Fig. 6c, arrowheads). Fractions 31–41 (Fig. 5a) showing a maximal GDPase and Ca^{2+} transport activity were enriched with cisterns and vesicles which look like the elements of the Golgi complex (Fig. 6d).

In conclusion, our data show that (i) the major part of both Ca^{2+}-transport and GDPase activities belongs to the fractions of membrane vesicles enriched with the Golgi structures, and (ii) that the Ca^{2+}-ATPase(s) plays the sole role in Ca^{2+} transport by total membrane vesicles.

This provides an evidence that, firstly, the predominant contribution to the Ca^{2+} sequestering is made by the Golgi

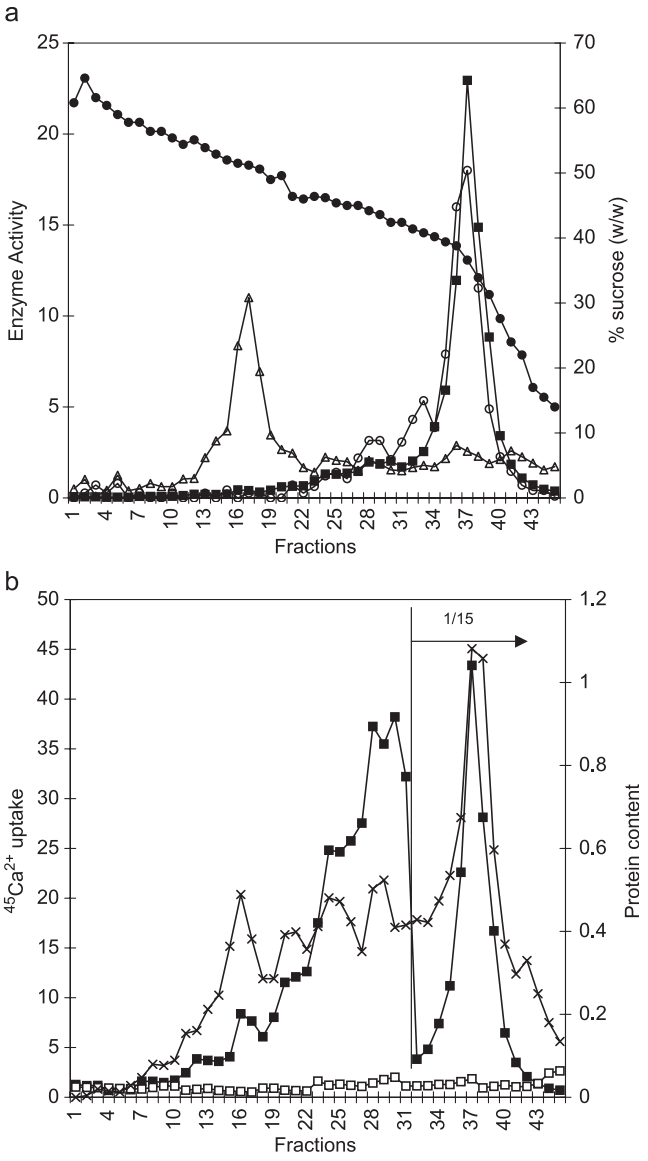

Fig. 5. (a) Separation of membrane vesicles of *T. foetus* on a sucrose density gradient. ATP-stimulated $^{45}Ca^{2+}$ uptake activity in the absence of oxalate (■) as well as the GDPase (○) and malic enzyme (△) activities of each fraction are expressed in percent from the total activities of all fractions taken as 100%. Note please that minor peaks of Ca^{2+} uptake activity of (b) are hardly seen here. Sucrose concentration, % (w/w), is shown by (●). One-hundred percent of GDPase activity corresponds to 2.1 μmol P_i/mg protein × 10 min^{-1} and 100% of the malic enzyme activity corresponds to 8.9 A_{340} units/mg × 10 min^{-1}. (b) Separation of the *T. foetus* membrane vesicles on a sucrose density gradient. $^{45}Ca^{2+}$ uptake activity (1000 cpm/20 μl samples) was determined in each fraction both in the presence of ATP (■) and in the absence of ATP (□). Protein content is presented in mg/ml of fraction (×). Note please that in fractions 32–45 the real $^{45}Ca^{2+}$ uptake activity is decreased by a factor of 15 to visualize better the minor peaks of $^{45}Ca^{2+}$ uptake activity of fractions 1–31.

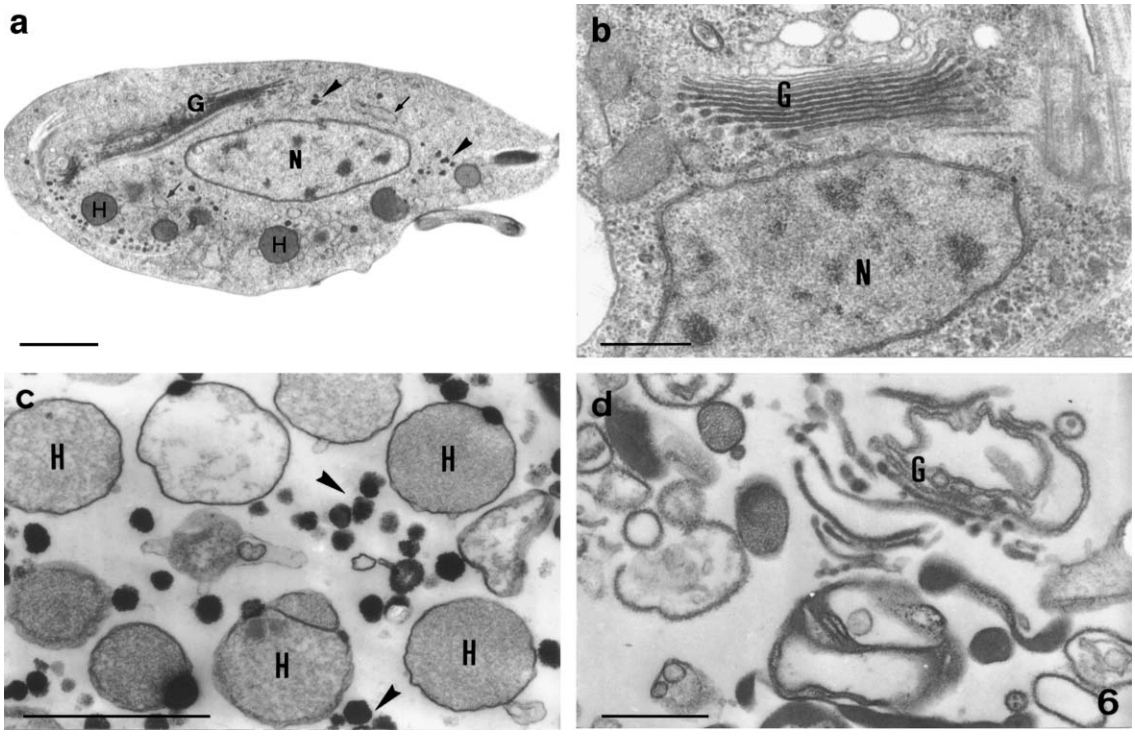

Fig. 6. Electron microscopy of *T. foetus* cells and isolated membrane fractions. (a) A typical view of thin section of *T. foetus* cell; the typical structures of the Golgi complex (G) close to the nucleus, hydrogenosomes (H) and glycogen granules (arrowheads) are seen. Vesicles of endoplasmic reticulum (arrows) are also visible. A Golgi complex can be seen in detail in (b). (c) Thin section of the membrane preparation of the peak which shows a high activity of malic enzyme (Fig. 5a). Note the presence of a large number of hydrogenosomes (H) and glycogen granules (arrowheads). (d) Thin section of the membrane preparation of the peak which shows a high GDPase and Ca^{2+} transport activity (Fig. 5a). The typical Golgi elements (G) can be observed. In (a) and (c), bar = 1 μm; in (b) and (d), bar = 0.5 μm.

and/or Golgi-like membrane vesicles of *T. foetus* and, secondly, that the Golgi membranes of this microorganism are equipped with Ca^{2+}-ATPase.

4. Discussion

Two main findings are described in this paper. The first one is that Ca^{2+} uptake by vesicles of total membranes isolated from *T. foetus* is only due to Ca^{2+}-ATPase activity and that Ca^{2+}/H^{+} antiporter does not contribute to the Ca^{2+} uptake. Moreover, Ca^{2+} uptake by membrane vesicles of all membrane fractions obtained after fractionation on a sucrose density gradient was not sensitive to FCCP (not shown), suggesting again a sole participation of Ca^{2+}-ATPase(s) in the formation and refilling of Ca^{2+} store pools in this protozoan. The presence of the nitrate-sensitive ATPase both in total membranes and in some membrane fractions isolated from *T. foetus* (not shown) indicated that membranes of this protozoan possess the H^{+} pump to provide energy to the Ca^{2+}/H^{+} antiporter. Given that the same method of the membrane isolation and fractionation revealed the significant activity of Ca^{2+}/H^{+} antiporter in the yeast *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* [5,31, and manuscript in preparation], we believe that this antiporter is not active in *T. foetus*. One can assume that the gene encoding the antiporter was not expressed in our experimental conditions or that the antiporter activity was inactivated during membrane isolation.

The second and very notable finding is that the Golgi complex is the dominating Ca^{2+} sequestering compartment of the *T. foetus* (Fig. 5). The main part of Ca^{2+}-ATPase activity of total membrane vesicles results from the enzyme of the Golgi. One can also note that the sensitivity of this enzyme to orthovanadate (I_{50} = 130 μM, Fig. 3) is equal to that of the yeast Golgi Ca^{2+}-ATPase (I_{50} = 130 μM; [24,32]) and the pea Golgi membranes (I_{50} = 126 μM , [35]). It is also similar to rabbit SERCA1 or SERCA2B [18].

Remarkably, the Golgi Ca^{2+}-ATPase of *T. foetus* is sensitive to thapsigargin (Fig. 4). In this respect, the Ca^{2+}-ATPase of *T. foetus* is similar to enzymes of the SERCA family [1,18,30] and to the Ca^{2+}-ATPase of the pea and cauliflower Golgi apparatus [35].

This indicates that Golgi, as the organelle of the secretory pathway of such ancient eukaryotic microorganism as *T. foetus* [33,34], is equipped with the enzyme similar to those of ER of animal cells and of plant Golgi [18,35].

Our data are in some contradiction with the report that the PMR1 Ca^{2+}-ATPase of the yeast Golgi is not sensitive to thapsigargin [32]. However, the reported insensitivity of

the yeast PMR1 Ca^{2+}-ATPase to thapsigargin may be a result of the low efficiency of the commercial preparation(s) of thapsigargin.

The role of different types of Ca^{2+} transporters in the Ca^{2+} sequestering in lower eukaryotes has been recently investigated by different groups. In the case of protozoa *T. cruzi* and *T. brucei*, Ca^{2+} transporters and Ca^{2+} sequestering organelles were analyzed using whole cells whose plasma membrane was permeabilized by treatment with digitonin or nystatin to make possible an access of intracellular organelles to extracellular substrates, ions, inhibitors and other reagents [7,8,10–12]. On the basis of these studies, Ca^{2+} transporters were classified according to the organelles they are presumably located in and which form different pools of the intracellular Ca^{2+}. The first pool is considered to be a mitochondrial one [7,12]. It is assumed that it is formed with Ca^{2+} uniporter, the function of which depends on the membrane potential (negative inside), because it was shown that a first component of Ca^{2+} uptake by the permeabilized cells was blocked by protonophores, ruthenium red and antimycin A [7,12]. The second pool was considered to be the ER pool because the other component of Ca^{2+} uptake was inhibited by orthovanadate. The third pool presumed to be composed of so-called "acidocalciosomes" [7,11,12].

The participation of Ca^{2+}/H^{+} antiporters and Ca^{2+}-ATPases in Ca^{2+} homeostasis of another eukaryotic microorganism, the yeast, was also investigated. It is widely accepted that the vacuolar Ca^{2+}/H^{+} antiporter can decrease Ca^{2+}concentration in the cytosol very rapidly [20–22] after its rise as a result of Ca^{2+} signaling [1–3]. However, it was also presumed that the antiporter cannot decrease a free Ca^{2+} to a low physiological concentration [2–4] since it has a low affinity for Ca^{2+} [20–22]. Therefore, Ca^{2+}-ATPase can then deplete the free Ca^{2+} concentration in cytosol because of the high affinity but low capacity of this transporter for Ca^{2+} [2–4].

It was shown that Ca^{2+}-ATPases and Ca^{2+}/H^{+} antiporter(s) operate simultaneously both in the vacuolar membrane and in membranes of various organelles of the secretory pathway of *S. cerevisiae* [5,6, and in preparation]. It is noteworthy that the fission yeast *Sch. pombe 972h*$^{-}$ grows well despite the apparent absence of any detectable activity of the Ca^{2+}-ATPases. Surprisingly, a Ca^{2+} sequestering in this yeast was shown to be carried out solely by Ca^{2+}/H^{+} antiporters energized by H^{+} gradient which was created by V type H^{+}-ATPases of different compartments of the secretory pathway [31].

In this respect our experimental data on exclusive participation of the Ca^{2+}-ATPase(s) in Ca^{2+} sequestering in *T. foetus* raise several questions which may lead to future investigations. The main question is how the gene expression of two types of Ca^{2+} transporters is controlled and/or how the activity of these transporters is regulated. The other question is: why can *T. foetus* use only the activity of Ca^{2+}-ATPases for its Ca^{2+} homeostasis, while *Sch. pombe* takes advantage of the Ca^{2+}/H^{+} antiporters, and *S. cerevisiae* and presumably *T. cruzi* and *T. brucei* can use both types of transporters? Why does not the facultative anaerobe *T. foetus* use H^{+} gradient for Ca^{2+} homeostasis and aerobic protozoa-like *T. cruzi* and *T. brucei* do? Can *T. foetus* express a gene(s) of the antiporter(s) when it is grown under other conditions? These and other questions need further experiments but it is clear that *T. foetus* offers a good opportunity for such investigation.

The finding of Ca^{2+} transport activity in different populations of membrane vesicles obtained by centrifugation on a sucrose density gradient (Fig. 5) is remarkable. Similar results were previously obtained for the *S. cerevisiae* and *Sch. pombe* yeast [5,6,31] as well as for plants *Ricinus comunis* and *Vigna unguiculata* (L.A.O, unpublished data). However, the contribution of different membrane fractions in the total Ca^{2+} sequestering in these species was more evenly distributed in comparison with the membranes of *T. foetus*, where the Golgi is the principal Ca^{2+} store compartment. (Fig. 5). The peak of Ca^{2+} transport activity which co-migrates with the activity of the malic enzyme, the marker of hydrogenosomes, probably results from a Ca^{2+}-ATPase of these organelles. It is also possible that the membrane vesicles of the rough ER partly contribute to the Ca^{2+} uptake by membrane vesicles of fractions 12–22 (Fig. 5). The participation of the plasma membrane vesicles in Ca^{2+} transport by total membrane vesicles is unlikely, or at least could not be significant because of (i) a low activity of the transporter in fractions 12–22 where plasma membrane can migrate and (ii) the relatively low sensitivity of the Ca^{2+} transport to orthovanadate (Fig. 3), since all known plasma membrane Ca^{2+}-ATPases are highly sensitive to orthovanadate [1]. However, we cannot rule out the participation of other compartments of *T. foetus* in Ca^{2+} storage despite their minor contribution to the total Ca^{2+} sequestering.

The fundamental contribution of Golgi membranes and their Ca^{2+}-ATPase(s) in Ca^{2+} transport by total membrane vesicles (80–85%, Fig. 5) and in total Ca^{2+} storage is an exceptional finding, at least for eukaryotic microorganisms. The only other known example of the predominant contribution of one compartment to Ca^{2+} sequestering is the sarcoplasmic reticulum of muscle cells which are highly differentiated [1].

In the *S. cerevisiae* and *Sch. pombe* yeast the contribution of various organelles of the secretory pathway to Ca^{2+} storage does not differ significantly [5,6,31]. It has been accepted that vacuole is the main Ca^{2+} store compartment of the yeast cell [3,4,19]. However, this assumption was based on experiments where an osmotically sensitive Ca^{2+} pool was equated with the vacuolar one and the contribution of the osmotically sensitive nonvacuolar organelles was not taken into consideration. Nonetheless, it was found that the yeast Golgi vesicles [24], as well as those of other yeast intracellular organelles, release Ca^{2+} as a result of the osmotic shock (unpublished data of L.A.O.). Our estimation of the contribution of yeast vacuoles to the Ca^{2+} storage

was based on the Ca^{2+} uptake capacity and showed that it varied from 15% to 45% depending on the yeast strain and its growth phase (5,6,31, and in preparation).

In conclusion, our data provide the evidence that the Golgi and its Ca^{2+} ATPase play a key role in the Ca^{2+} sequestering in *T. foetus*. It is tempting to speculate that well-developed Golgi complex in this protozoan [36,37] together with its major role in Ca^{2+} sequestering might be a result of its adaptation to a parasitic way of life.

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