Functional difference between SERCA2a and SERCA2b Ca^{2+} pumps and their modulation by phospholamban

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COS 1 cells were transfected with full-length pig stomach sarcoplasmic/endoplasmic reticulum Ca^{2+} pump (SERCA)2a or SERCA2b cDNA. Ca^{2+} uptake by microsomes from transfected cells revealed that the Ca^{2+} affinity of the SERCA2b Ca^{2+} pump ($K_{0.5}$ $0.17 \pm 0.01 \mu\text{M}$) was higher than that of the SERCA2a Ca^{2+} pump ($K_{0.5}$ $0.31 \pm 0.02 \mu\text{M}$). Thapsigargin-sensitivity was found to be identical for the two isoforms. The Ca^{2+} affinity of both the SERCA2a and SERCA2b Ca^{2+} pumps was decreased by a factor of two when they were co-expressed with phospholamban.

INTRODUCTION

Ca^{2+} accumulation into intracellular stores is mediated by Ca^{2+} -transport ATPases of the SERCA (sarco/endoplasmic reticulum Ca^{2+} -ATPase) family. So far, three different mammalian SERCA genes have been reported: *SERCA1*, *SERCA2* and *SERCA3* [1–6]. Alternative splicing of the *SERCA2* gene transcript gives rise to two distinct protein isoforms: SERCA2a, the muscle isoform, is expressed in slow skeletal muscle, heart and to a limited extent also in smooth muscle; SERCA2b, the non-muscle isoform, is expressed in smooth muscle and non-muscle tissues. The last four amino acids of the SERCA2a protein are replaced by an extended tail of 49 amino acids in the SERCA2b ATPase.

The SERCA Ca^{2+} -transport ATPases are modulated by phospholamban (PLB), a small homopentameric [7] sarcoplasmic reticulum/endoplasmic reticulum (SR/ER) membrane protein (52 amino acids; M_r 6000) which is expressed in cardiac muscle, smooth muscle and slow skeletal muscle, but not in fast skeletal muscle [8–10]. Phosphorylation of PLB in cardiac SR vesicles by cyclic AMP-, cyclic GMP- or Ca^{2+} /calmodulin-dependent protein kinases [11–13] raises the apparent affinity of the pump for Ca^{2+} by a factor of two. Indeed, it has been demonstrated that non-phosphorylated PLB acts as an inhibitor of the Ca^{2+} pump and that this inhibition can be reversed upon phosphorylation of PLB [14,15]. Cross-linking experiments of cardiac SR Ca^{2+} -transport ATPase and PLB suggested a direct interaction of SERCA2a in the Ca^{2+} -free conformation with non-phosphorylated PLB [16,17]. The PLB-binding peptide sequence in these SR Ca^{2+} pumps is located immediately C-terminal to the phosphorylation site [16]. Co-expression experiments of SERCA1 or SERCA2a with PLB confirmed their interaction [18], but no such data were available for SERCA2b.

The rate of Ca^{2+} uptake in smooth-muscle ER vesicles is stimulated to the same extent by cyclic AMP-dependent and cyclic GMP-dependent protein kinases, but this stimulation appears to be smaller than that seen in cardiac SR controls [19]. In cardiac muscle only the SERCA2a isoform is expressed. In contrast, smooth muscle contains 70–80% SERCA2b, and the remainder is SERCA2a [20]. Hence it was of interest to examine whether PLB interacts with the SERCA2a and 2b isoforms to a similar extent.

Recently Sagara *et al.* [21] reported that thapsigargin inhibits SERCA Ca^{2+} -transport ATPase activity by binding to and

stabilizing the Ca^{2+} pump in its Ca^{2+} -free state. This inhibition is due to a high-affinity 1:1 stoichiometric interaction between the inhibitor and the ATPase which is specific for SERCA-type Ca^{2+} pumps [22].

In the present study, microsomes were prepared from COS 1 cells transfected with SERCA2 cDNA encoding either the a or the b isoform. In some experiments the cells were co-transfected with PLB cDNA. Ca^{2+} -transport assays revealed that the apparent affinity of SERCA2b for Ca^{2+} is higher than that of SERCA2a. No difference in thapsigargin-sensitivity was measured for the two isoforms. Moreover, as for SERCA2a, the Ca^{2+} affinity of SERCA2b decreases when it is co-expressed with PLB.

MATERIALS AND METHODS

Vector construction and transfection of COS 1 cells

The cDNAs encoding the full-length pig stomach SERCA2a and SERCA2b isoforms [3] and the pig stomach PLB-8 cDNA clone [23] were each ligated in the unique *EcoRI* site of the mammalian expression vector pSV57 [24,25], in which expression is controlled by the late simian virus 40 (SV40) promoter. This vector was kindly provided by Dr. D. Huylebroeck, INNOGENETICS, Ghent, Belgium. Transfection of COS 1 cells was done in serum-free medium [26] by the DEAE-dextran method [27], using 7.5 μg of vector construct per 10 cm dish. For co-transfections, 7.5 μg each for both vector constructs was used per 10 cm dish. The day before transfection, cells were seeded at a density of 3×10^4 cells/cm².

Preparation and analysis of the microsomal fraction

At 60 h after transfection, the transfected cells were washed twice with 3 ml of phosphate-buffered saline (PBS) [which contained (in mM) 3.5 KCl, 1.4 KH_2PO_4 , 136 NaCl and 8.1 Na_2HPO_4], and subsequently scraped off the Petri dish into PBS supplemented with 5 mM-EDTA, 0.1 mM-phenylmethanesulphonyl fluoride (PMSF) and 100 units of aprotinin/ml. The harvested cells were washed once in PBS and allowed to swell on ice for 10 min in a hypo-osmotic solution containing 10 mM-Tris/HCl and 0.5 mM- MgCl_2 at pH 7.5. Subsequently the cells were homogenized in the presence of 0.1 mM-PMSF and aprotinin (100 units/ml) using a tight-fitting glass Dounce homogenizer (30 strokes). An equal volume of a solution containing 0.5 M-sucrose, 6 mM-2-mercaptoethanol, 40 μM - CaCl_2 , 300 mM-KCl and 10 mM-Tris/HCl (pH 7.5) was added to

Abbreviations used: SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} pump; PLB, phospholamban; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; PMSF, phenylmethanesulphonyl fluoride; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.

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the homogenate to re-establish iso-osmoticity. This homogenate was centrifuged at $1000 g_{\max}$ for 10 min to sediment the nuclei and the non-homogenized cells and the supernatant were further centrifuged at $200\,000 g_{\max}$ for 30 min. The resulting pellet was resuspended in 0.25 M-sucrose, quickly frozen in liquid N_2 and stored at $-70^\circ C$.

The microsomal protein concentration was determined by the enhanced protocol of the bicinchoninic acid method of Pierce (Rockford, IL, U.S.A.) using BSA as a standard [28]. SDS/PAGE was done on 0.75 mm-thick Laemmli-type slab gels containing 12% (w/v) polyacrylamide. Western blotting on Immobilon P membranes (Millipore) was carried out by the method of Towbin *et al.* [29]. For the immunodetection of SERCA2a and SERCA2b we used the non-discriminating monoclonal antibody IID8, kindly provided by Dr. K. P. Campbell (Howard Hughes Medical Institute, University of Iowa, IA, U.S.A.). For the immunodetection of PLB we used monoclonal antibody IID9, kindly provided by Dr. L. R. Jones (Krannert Institute of Cardiology, Indianapolis, IN, U.S.A.).

Estimation of the amount of Ca^{2+} -pump protein in the microsomal fraction was done by comparing the intensity of the signal on immunoblots with the signals of increasing amounts of a cardiac SR preparation. The amount of Ca^{2+} -ATPase relative to the total protein content of the cardiac reference samples was determined from densitometric scanning of Coomassie Blue-stained Laemmli gels.

Ca^{2+} -transport assay

Ca^{2+} uptake was measured by a rapid filtration technique at $37^\circ C$ in a medium containing 30 mM-imidazole, 0.1 M-KCl, 5 mM-ATP, 0.5 mM-KEGTA, 5 mM-potassium oxalate, 5 mM- NaN_3 , 1 mM free Mg^{2+} , ^{45}Ca at $2.5\text{--}4.0 \mu Ci/ml$ and different free Ca^{2+} concentrations at pH 6.8. Radioactivity was measured by liquid scintillation counting. Free Ca^{2+} concentrations were calculated as in [19].

Immunocytochemistry

Cells were seeded in coverglass chambers (Nunc, Naperville, IL, U.S.A.) at a density of 1.5×10^4 or $3.0 \times 10^4/cm^2$, and transfected as described above. At 2 days after transfection, the cells were fixed in freshly prepared 4% paraformaldehyde in PBS and permeabilized for 20 min in 0.25% Triton X-100 in PBS, pH 7.4. Immunostaining was done by a modification of the indirect immunofluorescence method described by Coons & Kaplan [30]. The monoclonal antibody IID8 and fluorescein isothiocyanate (FITC)-labelled goat anti-mouse antibodies (Sigma) were used to detect the SERCA2a and 2b Ca^{2+} pumps. The monoclonal antibody IID9 and phycoerythrin-labelled goat anti-mouse antibodies (Sigma) were used for detection of PLB. In the negative control experiments the primary antibodies were omitted. Staining was analysed with an ACAS 570 confocal laser microscope (Meridian, Okemos, MN, U.S.A.). The cells were illuminated ($100 \times$ oil objective; Olympus NA 1.3) by the 488 nm line of the tunable 5 W argon ion u.v./visible laser (Coherent, I-90). FITC fluorescence was monitored through a 530 nm interference filter (band pass 30 nm), and phycoerythrin fluorescence was monitored through a 575 nm long-pass filter. Confocal image scans of $360 \mu m \times 360 \mu m$ (for determining the amount of cells) or $72 \mu m \times 72 \mu m$ (for the analysis of individual cells) with a thickness of $0.5 \mu m$ were obtained.

RESULTS

Microsomes from COS 1 cells transfected with $7.5 \mu g$ of SERCA2b cDNA per 10 cm dish displayed a more than 10-fold increase of the SERCA2b protein as compared with control

microsomes (Fig. 1). The PLB/ATPase ratio in microsomes from co-transfected cells was similar to that seen in the cardiac SR preparation. We have estimated that, depending on the passage number of the COS cells, the exogenous pump protein amounted to between 0.25 and 1.2% of the microsomal protein content. Immunocytochemical analysis (18 image scans) showed that

Fig. 1. Expression of pig PLB and SERCA2b protein in COS 1 cells

Microsomal proteins ($50 \mu g$) prepared from control or transfected COS cells and $35 \mu g$ of cardiac SR microsomal proteins were subjected to SDS/PAGE (12% gels), Western blotting and immunodetection as described in the Materials and methods section. Control microsomes were prepared from COS cells which received the same treatment as transfected cells, but without DNA. Lane 1, control microsomes; lane 2, microsomes from COS cells transfected with pig stomach SERCA2b cDNA; lanes 3 and 4, microsomes from COS cells transfected with pig stomach PLB and SERCA2b cDNA; lanes 5 and 6, cardiac SR microsomes. +, with boiling; -, without boiling. The arrowhead indicates an aspecific binding.

Fig. 2. Time-dependent Ca^{2+} uptake by COS 1 microsomes

Oxalate-stimulated Ca^{2+} uptake was measured at $3.6 \mu M$ free Ca^{2+} and was stopped after 2, 10 and 20 mins as described in the Materials and methods section. Samples of $30\text{--}50 \mu g$ of microsomal proteins were used per ml of uptake medium. The results are the means of six measurements done on three independent membrane preparations; error bars represent s.e.m. Closed symbols, measurements with ATP; open symbols, measurements without ATP. $\blacktriangle$, $\triangle$, Microsomes from COS cells transfected with pig stomach SERCA2b cDNA; $\blacksquare$, $\square$, microsomes from COS cells transfected with pig stomach SERCA2a cDNA; $\bullet$, $\circ$, control microsomes.

Fig. 3. Ca²⁺-dependence of Ca²⁺ uptake by SERCA2a and SERCA 2b

Ca²⁺ uptake was measured at 0.03, 0.06, 0.11, 0.22, 0.36, 0.69, 1.22 and 3.6 μM free Ca²⁺ and stopped after 5 min as described in the Materials and methods section. At higher free Ca²⁺ concentrations, uptake values decreased. Samples of 30–50 μg of microsomal protein were used per ml of uptake medium. The results are the means of eight measurements, done on four independent membrane preparations; error bars represent S.E.M. $\circ$ Microsomes from COS cells transfected with pig stomach SERCA2a cDNA; $\bullet$, microsomes from COS cells transfected with pig stomach SERCA2b cDNA.

under our standard transfection conditions about 5% of the COS cells expressed the exogenous protein. Staining of PLB (17 image scans) as well as of the two ATPase isoforms (SERCA2a, 10 image scans; SERCA2b, 19 image scans) was observed throughout the whole cytoplasm. The distribution of these intrinsic membrane proteins is probably confined to the ER/Golgi membranes. Dual labelling and microscopical analysis of co-transfected cells showed that 90% of the cells which expressed the Ca²⁺ pump also expressed PLB (16 image scans; results not shown).

Microsomal fractions from COS cells transfected with SERCA2a and 2b cDNA displayed a 3–4-fold higher rate of ATP-dependent Ca²⁺ uptake than the control microsomes (Fig. 2). The efficiency of transfection decreased with increasing passage number of the COS cells. For the microsomal fraction used in the experiment illustrated in Fig. 2, we have estimated that the content of the exogenous ATPase was about 0.3% of the microsomal protein.

In a number of experiments SERCA2a and SERCA2b were expressed in parallel to similar levels. Fig. 3 shows the Ca²⁺-dependence of the Ca²⁺-uptake activity for both Ca²⁺-pump isoforms. The kinetic parameters are derived from the Hill equation. The $K_{0.5}$ value for SERCA2b was $0.17 \pm 0.01 \mu\text{M}$ ($n = 8$), which is significantly lower than the value obtained for SERCA2a ($0.31 \pm 0.02 \mu\text{M}$; $n = 8$). Because the only structural difference between SERCA2a and SERCA2b proteins is confined to the extended C-terminus of SERCA2b, the latter must be responsible for the higher Ca²⁺ affinity of SERCA2b.

Thapsigargin was found to be a powerful inhibitor of both Ca²⁺-pump isoforms. The Ca²⁺-uptake rate decreased with an IC_{50} of $4.8 \pm 0.3 \text{ nM}$ for SERCA2a, whereas an IC_{50} value of $3.4 \pm 0.1 \text{ nM}$ was obtained for SERCA2b. As shown in Fig. 4, Ca²⁺ uptake was almost completely blocked by 20 nM of this drug. As pointed out by Lytton *et al.* [22], the affinity of thapsigargin for the SERCA pumps is extremely high and the inhibition curves rather represent the titration of the Ca²⁺-pump protein by thapsigargin. The similar IC_{50} values for SERCA2a

Fig. 4. Inhibition of (a) SERCA2a and (b) SERCA2b by thapsigargin

Microsomal proteins from transfected COS cells (50 $\mu\text{g}/\text{ml}$ of uptake medium) were preincubated for 5 min with 0.2, 0.5, 1.0, 5.0 or 20.0 nM-thapsigargin. Ca²⁺ uptake was measured at 3.6 μM free Ca²⁺ and stopped after 5 min as described in the Materials and methods section. The results are the means of three measurements on one membrane preparation; error bars represent S.E.M.

and SERCA2b are compatible with their very similar expression levels.

Fig. 5 shows that in the presence of PLB the $K_{0.5}$ value for SERCA2a was almost doubled ($0.61 \pm 0.01 \mu\text{M}$ free Ca²⁺ versus $0.34 \pm 0.01 \mu\text{M}$ free Ca²⁺ in control). These experiments confirm the earlier report of Fujii *et al.* [16]. Furthermore, it is shown that the apparent affinity of the SERCA2b protein was also lowered by a factor of two when it was co-expressed with PLB. The K_m value for SERCA2b was $0.17 \pm 0.01 \mu\text{M}$, while in the presence of PLB a K_m value of $0.31 \pm 0.01 \mu\text{M}$ was obtained.

DISCUSSION

We have transfected COS cells with cDNA encoding the full-length pig stomach SERCA2a or SERCA2b protein and measured Ca²⁺ uptake in microsomal fractions prepared from these cells. Judging from the immunoblotting experiments, transfected cells displayed a 10-fold increase in the level of Ca²⁺-pump molecules, and yet only a 3–4-fold increase in Ca²⁺-uptake activity (Fig. 2). This discrepancy can be explained by the fact that the immunological estimation of the expression level requires only a structurally intact epitope, whereas the Ca²⁺-transport activity also requires a functional intact ATPase and structural integrity of the vesicles. Initial rates were estimated from ⁴⁵Ca²⁺ uptake after 5 min. Control experiments showed that the time course, as well as the V_{max} value of ⁴⁵Ca²⁺ uptake when

Fig. 5. Effect of PLB on Ca^{2+} uptake by SERCA2a and SERCA2b

Ca^{2+} uptake was measured at different free Ca^{2+} concentrations and stopped after 5 min as described in the Materials and methods section. Microsomal proteins were used at 50 $\mu\text{g}/\text{ml}$ of uptake medium. Results are means of six measurements, done on three independent membrane preparations; error bars represent S.E.M. (a), ●, Microsomes from COS cells transfected with pig stomach SERCA2a cDNA; ○, microsomes from COS cells co-transfected with pig stomach PLB and SERCA2a cDNA. (b), ●, Microsomes from COS cells transfected with pig stomach SERCA2b cDNA; ○, microsomes from COS cells co-transfected with pig stomach PLB and SERCA2b cDNA.

determined at this time point, were not significantly different between microsomes of cells transfected with either SERCA2a or SERCA2b (Fig. 2).

The Ca^{2+} -uptake assays on microsomal fractions from transfected cells revealed that the $K_{0.5}$ (Ca^{2+}) values for the two isoforms differ, amounting to $0.17 \pm 0.01 \mu\text{M}$ free Ca^{2+} and $0.34 \pm 0.01 \mu\text{M}$ free Ca^{2+} for SERCA2b and SERCA2a respectively. In a similar approach with the fast-twitch Ca^{2+} -transport ATPase (SERCA1) isoforms, which also present isoform diversity in their C-terminal end, Maruyama & MacLennan [31] were unable to find any functional differences. SERCA1a (the adult isoform) has a C-terminal glycine which in SERCA1b (the neonatal isoform) is replaced by a highly charged octapeptide [1,2]. Both have an identical $K_{0.5}$ value of $0.2 \mu\text{M}$ free Ca^{2+} .

For the plasma-membrane Ca^{2+} pump, Falchetto *et al.* [32] reported evidence for an intramolecular interaction between the calmodulin-binding domain and a region close to the active site of the pump, i.e. immediately C-terminal to the aspartyl-phosphate autophosphorylation site. They proposed that the calmodulin-binding domain acts as an endogenous inhibitor that can be switched off upon calmodulin binding. In analogy with

these results, it is tempting to suggest the occurrence of an intramolecular interaction in the case of the SERCA2b protein, in which the extended tail could serve as an endogenous stimulator. Alternatively, it is also possible that the extended tail interacts with one or more of the transmembrane helices responsible for Ca^{2+} binding, thereby enhancing the affinity of the high-affinity binding sites for Ca^{2+} . Indeed, the extended C-terminal segment of the SERCA2b protein contains a stretch of 20 uncharged hydrophobic amino acids, which might constitute an additional transmembrane region [4,5] and is followed by an adjacent hydrophilic segment. However, it has not as yet been experimentally verified whether this hydrophobic stretch is indeed membrane-spanning.

Our observation that the SERCA2a and 2b proteins show the same thapsigargin-sensitivity agrees with the recent report of Lytton *et al.* [22]. As also suggested by these investigators, these data imply that the thapsigargin-binding site is not located on the hydrophobic C-terminus which is unique to the SERCA2b protein. In the experiments on thapsigargin-sensitivity we used 50 μg of microsomal proteins from transfected COS cells per ml of uptake medium, corresponding to roughly 10 nM of ATPase. Ca^{2+} -uptake activity for both isoforms was almost completely blocked by 20 nM-thapsigargin. The apparent affinity of thapsigargin, which probably initially distributes preferentially in the hydrophobic membrane phase, is high enough to actually titrate the available ATPase molecules. As pointed out by Lytton *et al.* [22], this could explain the apparent discrepancy with earlier results [33] which erroneously had suggested a lower thapsigargin-sensitivity for the SERCA2a compared with SERCA2b. While the present work was in progress, similar conclusions were drawn by Sagara & Inesi [21] concerning the SERCA1 pump. They also found the thapsigargin inhibition to be dependent on the ATPase protein concentration. In their experiments, 1 μg of the ATPase present in fragmented skeletal-muscle longitudinal SR was inhibited by 1–10 nM-thapsigargin. As the Ca^{2+} pump represents 70–80% of the skeletal-muscle SR proteins, this finding correlates well with our results. Although the effective binding constants cannot be derived from these studies, it may be concluded that thapsigargin has a very high affinity for SERCA1 as well as for SERCA2.

We have expressed SERCA2a and SERCA2b proteins together with PLB in COS 1 cells in order to compare the effect of PLB on the apparent Ca^{2+} affinity of both isoforms. Immunodetection on Western blots showed that in our co-transfected COS cells the PLB/SERCA2a or PLB/SERCA2b ratios were comparable with the PLB/ATPase ratio in heart cells (Fig. 1). It is clear that PLB, when co-expressed with either isoforms, increases its $K_{0.5}$ for Ca^{2+} uptake. This observation also shows that the SERCA2b pump interacts with PLB. This is not surprising, because the alleged target site for PLB binding is represented in both isoforms [17], but it excludes the possibility that the 2b-specific tail interferes with the PLB-ATPase interaction. The stimulation of the rate of Ca^{2+} uptake by cyclic AMP- and cyclic GMP-dependent protein kinase in smooth-muscle ER vesicles has been found to be smaller than that in cardiac SR vesicles [19]. This relatively lower effect of PLB in smooth muscle cannot be explained by a lower expression level of PLB. Indeed, several types of smooth muscle express similar PLB/SERCA2 ratios to those in cardiac muscle [20]. Neither can it be explained by PLB isoform diversity, as cDNA sequencing has shown that smooth-muscle PLB is identical to cardiac or slow-skeletal-muscle PLB [23]. The present results furthermore also discount the possibility that a SERCA2 isoform diversity is responsible for the relatively lower PLB effect in smooth muscle. A difference in the phospholipid composition of the smooth-muscle versus cardiac-muscle membranes could be envisaged. Evidence has been reported for

a possible role of phosphorylated phospholipids in addition to PLB phosphorylation in the stimulation of the Ca²⁺-ATPase in cardiac SR [34].

In summary, our data suggest that the extended C-terminal tail of the SERCA2b Ca²⁺ pump may be responsible for its higher Ca²⁺ affinity compared with the truncated SERCA2a variant. The interaction with the SERCA pump inhibitor thapsigargin and the interaction with PLB are obviously not related to the SERCA2b-specific tail, and therefore cannot discriminate between the two isoforms.

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