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Rat vas deferens SERCA2 is modulated by Ca^{2+}/calmodulin protein kinase II-mediated phosphorylation

J.B.R. Rodriguez[1], H. Muzi-Filho[1], R.H.F. Valverde[2], L.E.M. Quintas[1], F. Noel[3], M. Einicker-Lamas[2,4] and V.M.N. Cunha[1]

[1]Programa de Farmacologia e Inflamação, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil
[2]Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil
[3]Programa de Desenvolvimento de Fármacos, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil
[4]Instituto Nacional de Ciência e Tecnologia em Biologia Estrutural e Bioimagem, Rio de Janeiro, RJ, Brasil

Abstract

Ca^{2+} pumps are important players in smooth muscle contraction. Nevertheless, little information is available about these pumps in the vas deferens. We have determined which subtype of sarco(endo)plasmic reticulum Ca^{2+}-ATPase isoform (SERCA) is expressed in rat vas deferens (RVD) and its modulation by calmodulin (CaM)-dependent mechanisms. The thapsigargin-sensitive Ca^{2+}-ATPase from a membrane fraction containing the highest SERCA levels in the RVD homogenate has the same molecular mass (~115 kDa) as that of SERCA2 from the rat cerebellum. It has a very high affinity for Ca^{2+} ($Ca_{0.5}$ = 780 nM) and a low sensitivity to vanadate (IC_{50} = 41 μM). These facts indicate that SERCA2 is present in the RVD. Immunoblotting for CaM and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) showed the expression of these two regulatory proteins. Ca^{2+} and CaM increased serine-phosphorylated residues of the 115-kDa protein, indicating the involvement of CaMKII in the regulatory phosphorylation of SERCA2. Phosphorylation is accompanied by an 8-fold increase of thapsigargin-sensitive Ca^{2+} accumulation in the lumen of vesicles derived from these membranes. These data establish that SERCA2 in the RVD is modulated by Ca^{2+} and CaM, possibly via CaMKII, in a process that results in stimulation of Ca^{2+} pumping activity.

Key words: Ca^{2+}/calmodulin-dependent kinase II; Calcium homeostasis; Calmodulin; Rat vas deferens; SERCA2; Thapsigargin

Introduction

The Ca^{2+} ion is one of the most important intracellular messengers in eukaryotic cells. After a triggering event, Ca^{2+} can be quickly mobilized from either the extracellular medium or internal stores, leading to diverse cell responses, including smooth muscle contraction (1,2). Most of the Ca^{2+} that enters the cytoplasm is rapidly bound to various cytosolic buffers (1). Calmodulin (CaM) is the most relevant Ca^{2+} binding protein and is also a sensor of alterations in intracellular Ca^{2+} concentration (3). This protein interacts reversibly with Ca^{2+} to form a Ca^{2+}/CaM complex, which can bind to different cellular targets. Indeed, many of the effects attributed to Ca^{2+} are exerted through Ca^{2+}/CaM-regulated enzymes (4). At the end of a Ca^{2+}-dependent cellular event, Ca^{2+} pumps embedded in the plasma membrane (PMCA) and in the membrane of the sarco(endo)plasmic reticulum (SERCA) actively transport Ca^{2+} ions from cytosol to the outside of the cells and to the lumen of the sarco(endo)plasmic reticulum, respectively (5,6).

The vas deferens is the tubule with contractile function essential for the ejaculation of sperm and hence male fertility. Experimentally, the rat vas deferens (RVD) has been extensively used in many physiological and pharmacological studies as a typical non-vascular smooth muscle tissue (7), representing an interesting model for investigation into the mechanisms of Ca^{2+} homeostasis (8). For instance, we have demonstrated that denervation of the RVD modifies the expression of several proteins

Correspondence: V.M.N. Cunha, Centro de Ciências da Saúde, UFRJ, Av. Carlos Chagas Filho, 373, Bloco J, 21941-902 Rio de Janeiro, RJ, Brasil. Fax: +55-21-2562-6659. E-mail: vcunha@farmaco.ufrj.br

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involved in intracellular Ca^{2+} regulation, including Na^+/K^+-ATPase, L-type Ca^{2+} channels and SERCA (9,10).

The SERCA family includes 3 gene products – SERCA1, SERCA2 and SERCA3 – that are expressed in a tissue-specific manner. Alternative splicing of the SERCA2 gene results in other different protein isoforms (11). The smooth muscle SERCA2b (115 kDa) isoform is identical to SERCA2a (110 kDa) except for its carboxyl terminus, where the four terminal amino acids of SERCA2a are replaced by an extended hydrophobic sequence of 49 amino acids. This stretch of amino acids presumably constitutes an 11th transmembrane segment for SERCA2b (12), which may be responsible for small biochemical and pharmacological differences between SERCA2a and SERCA2b isoforms (13). Activation of Ca^{2+}/CaM-dependent protein kinase II (CaMKII) stimulates the activity of SERCA2a pumps by direct phosphorylation of the Ser38 residue in these isoforms (14,15). In addition, CaMKII can phosphorylate phospholamban (16), a well-known modulator of SERCA pumps (17), and ryanodine receptor-Ca^{2+} release channels (18).

A 115-kDa SERCA isoform is present in the RVD (19), but its biochemical and regulatory properties have yet to be determined. We have shown (20) that, among all subcellular fractions of RVD, the nuclear fraction contains the highest content of thapsigargin-sensitive Ca^{2+}-ATPase activity (SERCA), with minor contamination of thapsigargin-resistant Ca^{2+}-ATPase activity (PMCA). Thus, this SERCA-enriched membrane fraction is more suitable for studies of sarcoplasmic reticulum components than the classical microsomal fraction. The objectives of the present study were to characterize the type of SERCA pump isoform in RVD as well as to investigate the ability of CaMKII to modulate SERCA in smooth muscle.

Material and Methods

Ethical considerations

All experimental procedures involving the animals were approved by the Committee for Ethics in Animal Experimentation of Universidade Federal do Rio de Janeiro, and were carried out in accordance with the Committee's guidelines.

Reagents and antibodies

The primary antibodies, anti-CaM and anti-CaMKII, were purchased from Sigma Chemical Company (USA) and anti-SERCA2 and anti-SERCA1 were purchased from Calbiochem-Novabiochem Co. (USA). Rainbow molecular weight markers were provided by GE Healthcare (UK); anti-P-Ser antibody was purchased from Biomol International (USA), and peroxidase-conjugated secondary antibodies were purchased from Promega Corporation (USA).

Calmodulin was purchased from Sigma Chemical Company. A23187 and thapsigargin were purchased from Calbiochem-Novabiochem Co. Stock solutions of 5 mM thapsigargin were prepared in 100% dimethyl sulfoxide (DMSO). At saturating concentrations of thapsigargin (3 μM) in buffers, the final concentration of DMSO was 0.07% (v/v), a concentration that had no effect on Ca^{2+}-ATPase activity or active Ca^{2+} uptake.

Preparation of a SERCA-enriched membrane fraction of RVD

Preparation of membranes was carried out as previously described (20). Briefly, RVDs were removed and immersed in cold Tyrode's solution containing 137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$ plus 0.36 mM NaH_2PO_4, pH 7.4, 5.55 mM glucose, 1.77 mM $CaCl_2$, and 0.40 mM $MgCl_2$. The tissue was dissected, homogenized and the crude homogenate was either centrifuged at 1000 *g* for 10 min to obtain the SERCA-enriched membrane fraction, or spun at 105,000 *g* for 60 min to obtain the whole homogenate fraction. The pellets were resuspended in Tris-HCl-buffered 0.25 M sucrose solution, pH 7.4, and stored in liquid N_2 until use (20). The protein content was determined by the method of Lowry et al. (21).

Measurements of Ca^{2+}-ATPase activity

SERCA-enriched subcellular samples of RVD (20 μg protein) were incubated for 2 h at 37°C in 0.5 mL medium containing 50 mM HEPES-Tris, pH 7.4, 10 mM NaN_3, 0.3 mM EGTA, 5 mM Na_2ATP, 4 mM $MgCl_2$, 5 μM A23187, 100 mM KCl and [γ-^{32}P]-ATP (specific activity: $\approx 1.5 \times 10^{10}$ Bq/mmol), with or without 3 μM thapsigargin, and in the presence or absence of different concentrations of $CaCl_2$ or vanadate (Na_3VO_4). The concentration of free Ca^{2+} was calculated according to Fabiato and Fabiato (22). Experiments were stopped by adding 1 mL of a cold mixture containing 26% (w/v) charcoal in 0.1 N HCl. The tubes were centrifuged at 1500 *g* at 4°C for 15 min, and 500 μL of the supernatant was placed onto filters. The filters were dried and the radioactivity was counted in a liquid scintillation counter. Ca^{2+}-ATPase activity was calculated by subtracting the basal ^{32}Pi release measured in the absence of Ca^{2+} (0.3 mM EGTA) from the total ^{32}Pi release measured in the presence of increasing free Ca^{2+} concentrations (EGTA.Ca^{2+} buffer) (20). The thapsigargin-resistant Ca^{2+}-ATPase activity (measured in the presence of thapsigargin) was subtracted from the total Ca^{2+}-ATPase activity to obtain the thapsigargin-sensitive Ca^{2+}-ATPase activity due to SERCA pumps (10,20). The Ca^{2+} dependence and the sensitivity of the enzyme to vanadate were measured over the ranges of 0.2 to 20 μM and 1 μM to 1 mM, respectively.

Measurements of $^{45}Ca^{2+}$ uptake

SERCA-enriched samples of RVD (50 μg protein) were incubated for 120 min at 37°C in a medium

containing 50 mM HEPES-Tris, pH 7.4, 10 mM NaN_3, 0.3 mM EGTA, 5 mM Na_2ATP, 4 mM $MgCl_2$, 100 mM KCl, with sufficient $CaCl_2$ to provide 10 μM free Ca^{2+} and $^{45}CaCl_2$ (specific activity: ≈1.5 × 10^9 Bq/mmol), in the presence or absence of 5 μM A23187. The reaction was stopped by vacuum filtration and the filters were washed twice with 20 mL cold 20 mM MOPS, pH 7.0, 2 mM $La(NO_3)_3$, and 100 mM KCl, and counted in a liquid scintillation counter. ATP-dependent Ca^{2+} accumulation in the lumen of vesicles derived from the membranes was calculated by subtracting the Ca^{2+} accumulation measured in the presence of A23187 (blank) from the total Ca^{2+} accumulation measured in the absence of the ionophore. The stimulatory effect of CaM was assayed by preincubating the membranes for 5 min in a reaction medium with or without 2 μM CaM (23,24) before the addition of Ca^{2+}. These experiments were done in the presence of ruthenium red (25 μM) to eliminate the stimulatory effect of CaMKII on Ca^{2+} release channels (24).

Immunodetection of SERCA, CaM and CaMKII

Western blotting was used to detect SERCA and to investigate whether CaM and CaMKII were present in the RVD-derived membranes. The samples were subjected to SDS-PAGE (6% polyacrylamide gel for SERCA1 and SERCA2, 7.5% for CaMKII and 15% for CaM) and transferred to nitrocellulose membranes. The membranes were incubated with 5% non-fat dry milk in Tris-buffered saline plus 0.1% Tween-20 followed by incubation with specific monoclonal (anti-CaM, 1:500 dilution; anti-SERCA1, 1:4000; anti-SERCA2, 1:3000) or polyclonal antibodies (anti-CaMKII, 1:1000) and with anti-mouse (CaM, 1:11,000; SERCA1 and 2, 1:12,000) or anti-rabbit (CaMKII, 1:10,000) horseradish peroxidase-conjugated secondary antibodies, with the blots being detected by chemiluminescence. In these assays, the heavy microsomal fraction of skeletal muscle from adult rats (25), rat cardiac microsomes (25), chicken cerebellum microsomes (26), and the membrane fraction from electrocytes of *Electrophorus electricus* (L.) (27) were used as positive controls for SERCA1, SERCA2a, SERCA2b, and CaMKII, respectively.

Immunodetection of serine-phosphorylated residues

The phosphorylation reaction was initiated by the addition of 0.8 mM cold ATP after preincubation of 5 μg of the SERCA-enriched fraction of RVD in a medium containing 50 mM HEPES-Tris, pH 7.4, 4 mM $MgCl_2$, 200 μM EGTA, and 100 mM KCl in the presence or absence of 10 μM free Ca^{2+} and 2 μM CaM at 4°C. The reaction was stopped after 2 min by adding 15 μL SDS sample buffer. The samples were run on 7.5% SDS-PAGE gels before being transferred to nitrocellulose membranes. Non-specific phosphorylation sites were blocked with 5% bovine serum albumin in Tris-buffered saline plus 0.1% Tween-20. Serine-phosphorylated peptides were detected using an anti-phosphoserine monoclonal antibody (1:500) and anti-mouse horseradish peroxidase-conjugated antibody (1:20,000). Rabbit skeletal muscle and rat cardiac microsomes were used as phosphoserine/phosphothreonine-positive controls (15). Control assays without Ca^{2+} and exogenous CaM, but in the presence of 0.8 mM exogenous ATP and without any additions (to detect any preexisting phosphorylation), were run in parallel.

Statistical analysis

Data are reported as means ± SE for 3 to 4 experiments performed in triplicate. Statistical comparisons were determined by one-way ANOVA. The differences were considered to be significant at $P < 0.05$. When Ca^{2+} concentration dependence and inhibition by vanadate were studied the equations were fitted to the experimental points by non-linear regression analysis (SigmaPlot, Jandell Scientific, USA).

Results

Ca^{2+}-ATPase activity from the SERCA-enriched membrane fraction of RVD: dependence on Ca^{2+} concentration and sensitivity to vanadate

To investigate the Ca^{2+} dependence and sensitivity to vanadate (a classical inhibitor of P-type ion motive ATPases) (28) of the Ca^{2+} pumps in SERCA-enriched membranes, the Ca^{2+}-ATPase activity was measured in the presence of increasing concentrations of Ca^{2+} or vanadate (in the presence of 10 μM free Ca^{2+} in the latter case). Thapsigargin-sensitive Ca^{2+}-ATPase activity, i.e., a SERCA-associated activity (29), increases with increasing Ca^{2+} concentration according to Equation 1.

$$v/V_{max} = [Ca^{2+}]/(Ca_{0.5} + [Ca^{2+}]) \quad \text{(Equation 1)}$$

where V_{max} and $Ca_{0.5}$ have the usual meaning (Figure 1). The thapsigargin-sensitive activity had a $Ca_{0.5}$ value of 780 nM and accounted for at least 80% of the total Ca^{2+}-ATPase activity (compare filled and open symbols). The thapsigargin-resistant activity was very low and, therefore, it was not possible to determine precisely $Ca_{0.5}$ or V_{max}. Vanadate inhibited the thapsigargin-sensitive Ca^{2+}-ATPase according to Equation 2 and Figure 2.

$$v_i/v_o = IC_{50}/\left(IC_{50} + \left[VO_4^{3-}\right]\right) \quad \text{(Equation 2)}$$

where v_o is the activity in the absence of vanadate, v_i is the activity at different inhibitor concentrations, and IC_{50} (41 μM) is the vanadate concentration required to attain 50% of the maximal inhibition.

Characterization of the SERCA isoform in RVD

To characterize the isoforms present in the SERCA-enriched membrane fraction and in the whole homogenate, Western blot assays were performed with mono-

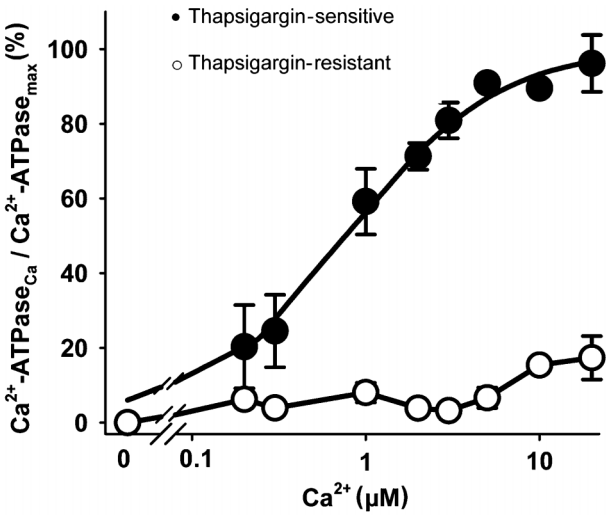

Figure 1. Ca^{2+} concentration dependence of thapsigargin-sensitive and -resistant Ca^{2+}-ATPase activity. The assays were performed in triplicate and data are reported as means ± SE for 3 experiments, using 3 different enzymatic preparations. The smooth curve was fitted to the thapsigargin-sensitive data points (filled circles) using Equation 1 as described in the text. Ca^{2+}-ATPase at 10 μM Ca^{2+} was 1.08 ± 0.11 μmol Pi x mg^{-1} in 2 h. Ordinate legend: Ca^{2+}-ATPase$_{Ca}$ and Ca^{2+}-ATPase$_{max}$ represent the activity at each Ca^{2+} concentration and the maximal activity obtained by fitting the function to the experimental points of thapsigargin-sensitive Ca^{2+}-ATPase values, respectively. Thapsigargin-resistant activities (open circles) were calculated from the total Ca^{2+}-ATPase activity at each Ca^{2+} concentration in the same assay.

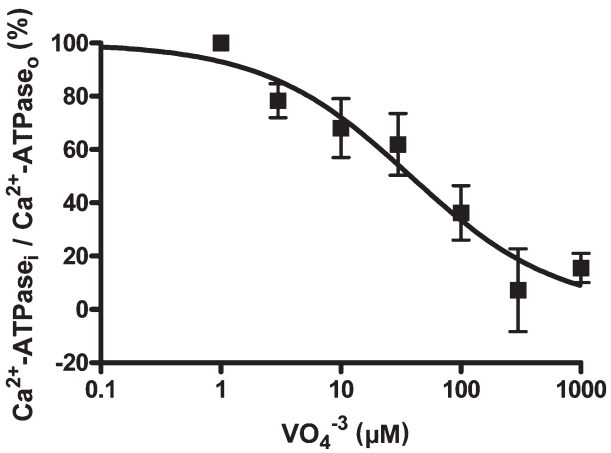

Figure 2. Inhibition of thapsigargin-sensitive Ca^{2+}-ATPase activity by vanadate. The assays were performed in triplicate with 10 μM Ca^{2+} and the results are reported as means ± SE for 4 experiments, using 3 different preparations. Ca^{2+}-ATPase activity was measured without vanadate in the control. The smooth curve was fitted to the data points using Equation 2 as described in the text. Ordinate legend: Ca^{2+}-ATPase$_i$ and Ca^{2+}-ATPase$_o$ represent activity in the presence and in the absence of vanadate at each concentration of the inhibitor, respectively.

clonal antibodies anti-SERCA1 or anti-SERCA2 pumps. The SERCA1 isoform was not detected in either the SERCA-enriched membrane or the whole homogenate (Figure 3A), whereas SERCA2 was the isoform present in the same preparations (lanes 1 and 2; Figure 3B). These bands have the same molecular mass as the positive control for SERCA2b (lane 3; chicken cerebellum, 115 kDa), but they also have a molecular mass near the SERCA2a band (lane 4; rat heart, 110 kDa). Figure 3 is a representative blot revealing the same profile that was found in the other two developed with the use of different RVD preparations.

Characterization of CaM and CaMKII from RVD

Western blotting analysis indicated a 17-kDa protein in the SERCA-enriched membrane fraction as well as in the crude homogenate of RVD that co-migrated with that found in an enriched CaM preparation (chicken cerebellum microsomes; Figure 4A shows a typical assay). Likewise, Figure 4B shows the presence of CaMKII (lanes 1-3) co-migrating with the positive controls (chicken cerebellum, lane 4; electrocyte membrane, lane 5) in the 50-kDa region. Faced with the abundance of these regulatory proteins in RVD, it is possible that both CaM and CaMKII modulate the SERCA pump isoform in RVD smooth muscle, stimulating a regulatory kinase-mediated phosphorylation that has been described in other tissues and species (14,29).

Phosphorylation of the SERCA2 pump from RVD by CaMKII

To investigate whether the SERCA2 pump from RVD is modulated by CaMKII (Figure 4B), phosphorylation assays were used to detect phosphoserine residues by Western blotting. A phosphorylated band of ~115 kDa was found in the SERCA-enriched fraction of RVD only after the addition

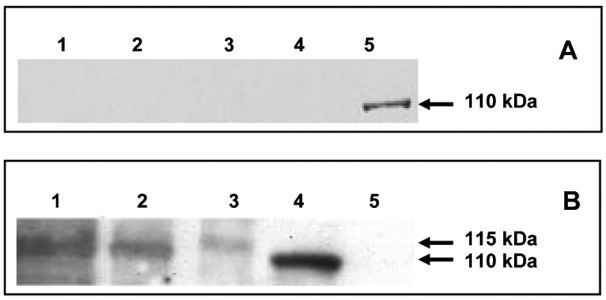

Figure 3. Expression of sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) isoforms. *A*, Immunoblotting with anti-SERCA1 antibody and *B*, immunoblotting with anti-SERCA2 antibody. *Lane 1* = 24 μg SERCA-enriched fraction; *lane 2* = 32 μg of crude homogenate; *lane 3* = 4 μg of chicken cerebellum microsomes (positive control for SERCA2b); *lane 4* = 10 μg of rat heart microsomes (positive control for SERCA2a); *lane 5* = 0.4 μg (in *A*) or 10 μg (in *B*) of heavy microsomes of rat fast skeletal muscle (positive control for SERCA1).

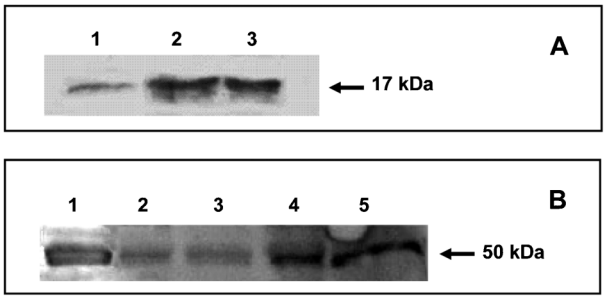

Figure 4. Expression of calmodulin (CaM) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) in the membranes of the SERCA-enriched fraction and crude homogenate. *A*, Immunoblotting with anti-calmodulin antibody. *Lane 1* = 24 μg of SERCA-enriched fraction; *lane 2* = 32 μg of whole homogenate; *lane 3* = 4 μg of chicken cerebellum microsomes (positive control for CaM). *B*, Immunoblotting with anti-CaMKII antibody. *Lanes 1, 2, 3* = 40 μg of 3 different SERCA-enriched fractions from RVD; *lane 4* = 4 μg of chicken cerebellum microsomes; *lane 5* = 20 μg of innervated fraction of membrane electrocyte of *Electrophorus electricus* (L.). *Lanes 4* and *5* are positive controls for CaMKII. SERCA = sarco(endo)plasmic reticulum Ca^{2+}-ATPase.

of Ca^{2+} and CaM (Figure 5), indicating that CaMKII phosphorylates serine residues of the SERCA2 pump.

Calmodulin stimulates active Ca^{2+} transport mediated by SERCA2 in RVD

Since CaM strongly stimulates kinase-mediated phosphorylation of SERCA2 (Figure 5), $^{45}Ca^{2+}$ uptake was assayed in the absence or presence of CaM to see whether CaMKII-mediated phosphorylation is associated with an increase in Ca^{2+} pumping activity (30). Depletion of CaM by mild alkaline treatment renders the vesicles leaky and Ca^{2+} accumulation is barely detectable. Therefore, we tested the influence of exogenous CaM on Ca^{2+} transport. Addition of CaM stimulates the steady levels of thapsigargin-sensitive

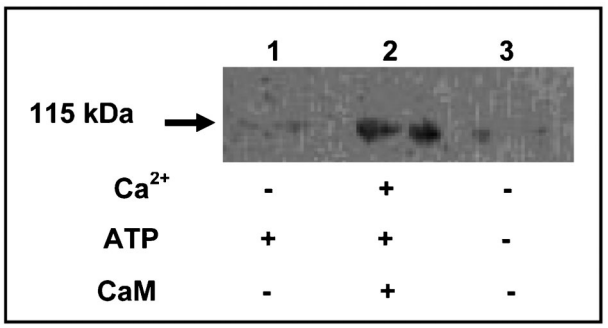

Figure 5. Phosphorylation of serine residues in sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA). Blots were obtained after incubation of phosphorylated membranes (5 μg) with anti-phosphoserine residue antibody. The phosphorylation assays were performed in the absence (*lane 1*) or presence (*lane 2*) of 10 μM free Ca^{2+} and 2 μM calmodulin (CaM). Control (*lane 3*) without Ca^{2+}, calmodulin and ATP shows no preexisting phosphorylation.

Ca^{2+} accumulation into the vesicles derived from the membranes by a factor of 8 (Figure 6).

Discussion

We have demonstrated that SERCA2 is present in subcellular membranes from RVD and that its pumping capacity is stimulated by CaM through a pathway that is probably mediated by CaMKII. In line with the results of our previous study (20), we have now worked with a so called ‘‘nuclear fraction’’ of RVD because i) it has a higher sarcoplasmic reticulum/plasma membrane ratio, and ii) the yield of thapsigargin-sensitive Ca^{2+}-ATPase is 4-fold higher when compared to the microsomal fraction, often chosen for these studies in several tissues/species (8,31).

SERCA pump isoforms have a high degree of sequence identity, but they can be reasonably distinguished from the type of tissue in which they are expressed by the analysis of biochemical parameters such as Ca^{2+} dependence, vanadate sensitivity, and by specific antibody recognition (13,32,33). Therefore, these criteria were used to characterize the SERCA isoform expressed in RVD. The Ca^{2+} affinity of SERCA isoforms reflects a critical functional property of Ca^{2+}-ATPases that is probably related to specialized Ca^{2+} environments (13,34). The SERCA2b isoform, localized in smooth muscle (19) (usually comprising >70% of total SERCA) (25), and non-muscle cells (19) has a 2-fold higher affinity

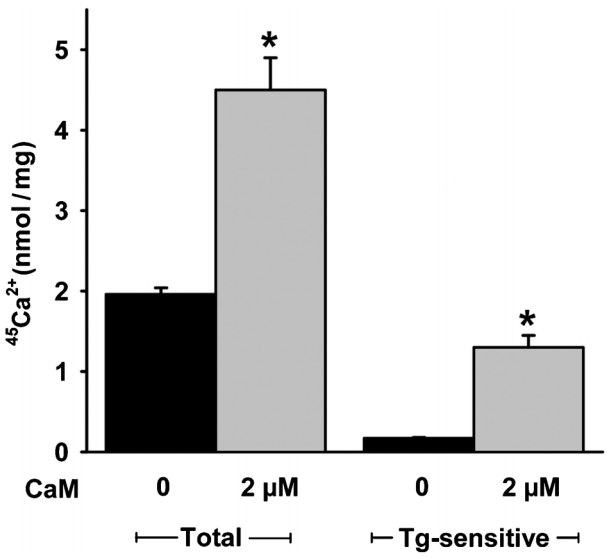

Figure 6. Effect of calmodulin (CaM) on total and thapsigargin (Tg)-sensitive $^{45}Ca^{2+}$ uptake by vesicle membranes from the SERCA-enriched fraction. $^{45}Ca^{2+}$ uptake without (black columns; controls) or with 2 μM exogenous calmodulin (gray columns). The assays were performed in triplicate and the data are reported as means ± SE for 4 experiments using 4 different preparations. SERCA = sarco(endo)plasmic reticulum Ca^{2+}-ATPase. *P < 0.05 compared to control (one-way ANOVA).

for Ca^{2+} ($Ca_{0.5}$ = 0.1-0.3 μM) compared to SERCA2a ($Ca_{0.5}$ = 0.2-0.5 μM) or SERCA1 (13,32,34), and 5-fold higher than SERCA3 ($Ca_{0.5}$ ~1 μM) (34), when expressed in COS or HEK-293 cells. Accordingly, these differences in Ca^{2+} affinity could be due to variations in the equilibrium between the two main conformations of the enzyme, E_1 and E_2 (12). Experiments in COS cells using SERCA2b mutants without the putative 11th transmembrane segment indicate that the apparently higher Ca^{2+} affinity of SERCA2b does not involve structural differences in Ca^{2+}-binding sites between SERCA2 isoforms, but instead would be a shift in the $E_1 \leftrightarrow E_2$ equilibrium towards the high-affinity E_1 state (13). The value of $Ca_{0.5}$ for the SERCA isoform in RVD (780 nM) is similar to that of the high-affinity SERCA2 isoform, even though these values are used to compare subcellular preparations similar to ours and preparations of cells transfected with isoforms from other species. The functional properties of the different SERCA isoforms expressed in cells may not be correlated exactly to the values obtained for native enzymes, since positive and negative modulators certainly are also expressed in the native environment (34), such as CaM and/or membrane-anchored CaMKII (27).

SERCA pumps are P-type ion motive ATPases and therefore they are inhibited by vanadate, a transition state analog of phosphate that specifically binds to and stabilizes the E_2 conformation (35). The differential sensitivity to vanadate is useful in distinguishing between the SERCA isoforms (13,34). Our results show that the thapsigargin-sensitive activity in RVD has a very low affinity for vanadate, with an IC_{50} of 41 μM compared to SERCA3 (~10 μM) (34). SERCA2b is less sensitive to vanadate when compared to SERCA2a in distinct expression systems, and the IC_{50} found falls near 100 μM for SERCA2b in other systems (25) Again, the decreased sensitivity to vanadate could be attributed to a shift in the equilibrium of the conformations toward E_1 (35), as in the case of the higher Ca^{2+} affinity discussed above. Thus, these data indicate that SERCA2b is possibly the isoform present in RVD.

Translational and post-translational regulation of SERCA2 isoforms has been well documented (17). Although it is well known that phospholamban phosphorylation increases the affinity of SERCA2 for Ca^{2+}, direct CaMKII-dependent phosphorylation of SERCA2 is also an important route to control the enzyme function (17,24,29). Before attempting to characterize this modulatory pathway in RVD, we checked for the presence of CaM and CaMKII in this tissue. Endogenous CaM was found in the SERCA-enriched fraction as well as in the crude homogenate. The same kind of experiment was performed for CaMKII. The polyclonal antibody employed recognizes all 4 CaMKII isoforms (α, β, γ, and δ) with molecular weights of 50-65 kDa (36), γ and δ being the more important isozymes in smooth muscles, with M_R of ~60 kDa (37). We demonstrated with this antibody that significant amounts of CaMKII are present in the SERCA-enriched fraction, which suggests a potential regulatory role for CaM/CaMKII in SERCA2 activity. This regulatory machinery was previously found in cardiac muscle, another tissue that co-expresses SERCA2, CaM and CaMKII.

In Western blotting assays for phosphorylated SERCA at serine residues, addition of Ca^{2+} and exogenous CaM promotes an intense phosphorylation at 115 kDa, i.e., at the M_R at which the antibody detected the Ca^{2+} pump. Therefore, this protein is clearly phosphorylated by CaMKII. However, phosphorylation was not related to the aspartyl-phosphorylated residue that is formed during the catalytic cycle of P-type ATPases, since the latter was unstable in the alkaline conditions used in the electrophoresis development (38). Even though the recognition of serine-phosphorylated SERCA would imply phosphorylation mediated by other kinases, the strong response to Ca^{2+} and CaM as well as the presence of high levels of CaMKII in the membranes support the idea that this enzyme is responsible for serine phosphorylation.

The addition of CaM in the presence of 10 μM free Ca^{2+} increases active Ca^{2+} accumulation by SERCA2-containing vesicles by one order of magnitude. This accumulation can only be measured in the presence of ruthenium red used to block the ryanodine receptors (Ca^{2+} release channels) activated by CaMKII (24). The huge increase in Ca^{2+} accumulation is not due to the binding of CaM to SERCA, since only PMCA has a CaM binding domain (39). Therefore, and in line with the strong increase in SERCA2 phosphorylation when Ca^{2+} and CaM are added together, we conclude that the increase in accumulated Ca^{2+} is due to CaMKII-mediated phosphorylation. CaMKII also directly or indirectly activates other Ca^{2+} pumping activities. For example, CaMKII-mediated phosphorylation of phospholamban activates cardiac SERCA2 through an increase in Ca^{2+} affinity (40). A direct CaMKII-mediated phospholamban-independent phosphorylation of SERCA2 results in enhanced maximal velocity of Ca^{2+} transport. However, the CaMKII dependent-phosphorylation of phospholamban cannot be selectively inhibited under the experimental conditions used (40). Although the presence of phospholamban has not been detected in RVD, we recently demonstrated the presence of a functional protein kinase A (PKA) in this tissue (Muzi-Filho H, Bezerra CGP, Souza AM, Boldrini LC, Takiya CM, Oliveira FL, et al. unpublished results). It is possible that PKA phosphorylates phospholamban increasing SERCA activity and directly phosphorylates PMCA isoforms of the RVD.

The present results show that the Ca^{2+} pump in RVD smooth muscle is SERCA2, possibly SERCA2b, as demonstrated by its high affinity for Ca^{2+}, low affinity for vanadate, and presence of the same molecular weight as the SERCA2b-positive control. This pump is co-expressed with CaM and CaMKII in the same membranes, is stimulated by the Ca^{2+}/CaM complex, and is

phosphorylated at specific serine residues. These observations support the hypothesis that the widely distributed (40) CaMKII-mediated phosphorylation could be a powerful regulatory mechanism of Ca^{2+} transport in RVD and its contractile activity.

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