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Functional Comparisons between Isoforms of the Sarcoplasmic or Endoplasmic Reticulum Family of Calcium Pumps*

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ATP-dependent calcium pumps that reside in intracellular organelles are encoded by a family of structurally related enzymes, termed the sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPases (SERCA), which each have a distinct pattern of tissue-specific and developmentally regulated expression. A COS-1 cell expression system was used to examine the biochemical properties of the isoforms: SERCA1 (fast-twitch skeletal muscle), SERCA2a (cardiac/slow-twitch skeletal muscle), SERCA2b (ubiquitous smooth- and non-muscle), and SERCA3 (non-muscle). Each isoform was expressed efficiently and appeared to be targeted to the endoplasmic reticulum. All isoforms displayed qualitatively similar enzymatic properties and were activated by calcium in a cooperative manner with a Hill coefficient of 2. The quantitative properties of SERCA1 and SERCA2a (the muscle isoforms) were identical in all respects. SERCA2b, however, appeared to have a lower turnover rate for both calcium transport and ATP hydrolysis. SERCA3 displayed a reduced apparent affinity for calcium, an increased apparent affinity for vanadate, and an altered pH dependence when compared with the other isoforms. These properties are consistent with an enzyme in which the equilibrium between the E_1 and E_2 conformations is shifted toward the E_2 state.

Cytoplasmic calcium acts as a ubiquitous messenger, controlling many different aspects of cellular physiology. Intracellular organelles play a critical role in this regulation by acting both as sites of storage for subsequent release and as sinks for removal of calcium from the cytoplasm. In striated muscle, calcium is stored principally within the terminal cisternae of the sarcoplasmic reticulum, is released through the calcium release channel (ryanodine receptor) to initiate contraction, and then taken back up into the sarcoplasmic reticulum via the well characterized Ca^{2+}-ATPase pump to allow relaxation (Lytton and MacLennan, 1991). In smooth- and non-muscle tissues, the calcium storage compartment is thought to be associated with the endoplasmic reticulum. Complex patterns of release catalyzed by a variety of agonists have led to the suggestion that these tissues possess at least two distinct calcium storage pools, one sensitive to caffeine and calcium (which stimulate release through the ryanodine receptor) and the other to inositol-1,4,5-trisphosphate (which stimulates calcium release through its own receptor) (Lytton and Nigam, 1992).

Biochemical data clearly demonstrate that non-muscle calcium stores are loaded by an ATP-dependent calcium pump, which bears much similarity to the enzyme of striated muscle sarcoplasmic reticulum (Spamer *et al.*, 1987). Molecular cloning studies have borne this out, and a family of sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPase (SERCA)[1] pumps has been identified (MacLennan *et al.*, 1985; Brandl *et al.*, 1986; Lytton and MacLennan, 1988; Gunteski-Hamblin *et al.*, 1988; Burk *et al.*, 1989). Although a correlation between different calcium storage pools and specific SERCA isoforms has not been established, studies with specific inhibitors have demonstrated an essential role for these enzymes in loading all agonist-sensitive stores (Lytton *et al.*, 1991a; Thastrup *et al.*, 1990; Bian *et al.*, 1991).

Three separate genes encode this family of calcium pumps. SERCA1 is expressed exclusively in fast-twitch skeletal muscle. Two developmentally regulated alternatively spliced species are transcribed from this gene, denoted "a" and "b," that encode protein products with different carboxyl termini (Brandl *et al.*, 1987). COS cell expression studies have not revealed a functional difference between the SERCA1a and b proteins, and thus the significance of these sequence differences is obscure (Maruyama and MacLennan, 1988).

The SERCA2 transcript was originally cloned from neonatal muscle and subsequently demonstrated to be expressed in both adult slow-twitch skeletal muscle and cardiac muscle at all stages of development (Brandl *et al.*, 1987). The protein product of this gene is about 84% identical to the SERCA1 protein. SERCA2 is also alternatively spliced in the region encoding the carboxyl terminus and produces two protein products. SERCA2a, which terminates in the sequence -Ala-Ile-Leu-Glu, is the major spliced species of this gene expressed in striated muscle. The other transcript, SERCA2b, encodes a protein in which the carboxyl-terminal four amino acids of SERCA2a are replaced by an extended, relatively hydrophobic, sequence of 49 or 50 amino acids. SERCA2b is ubiquitously expressed and appears to represent a generic "endo-

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[1] The abbreviations used are: SERCA, sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPase; DMEM, Dulbecco's modified Eagle's medium containing high glucose (4.5 g/l) and pyruvate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; *E*-P, the covalently phosphorylated enzyme-intermediate of the Ca^{2+}-ATPase reaction cycle; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

plasmic reticulum" form of the Ca^{2+}-ATPase (Lytton *et al.*, 1989).

A third gene, SERCA3, encoding a protein about 75% identical to either the SERCA1 or SERCA2 products, was also identified in a variety of non-muscle tissues, although its expression is not as ubiquitous as SERCA2b (Burk *et al.*, 1989). The sequence differences between SERCA3 and the other SERCA isoforms are clustered at the amino terminus, in regions of the nucleotide-binding domain, and at the carboxyl terminus. Interestingly, SERCA3 contains a unique sequence of 15 amino acids at its carboxyl terminus which is remarkably similar to the same region of the adenovirus E3/19K protein demonstrated to provide the signal for retention in the endoplasmic reticulum, suggesting that SERCA3 may also be localized to the endoplasmic reticulum.

As a consequence of their conserved primary structure, all of the known SERCA isoforms are predicted to have essentially identical transmembrane topologies and tertiary structures. Extensive site-directed mutagenesis studies of the SERCA1 protein have defined many residues which are critical for normal functioning of the enzyme and pump; these are all conserved among the different SERCA isoforms (MacLennan, 1990; Lytton and MacLennan, 1991). Not surprisingly, each isoform has been demonstrated to transport calcium in an ATP-dependent fashion. Furthermore, all of the SERCA isoforms are inhibited by the sesquiterpene lactone, non-phorboid tumor promoter, thapsigargin, although this agent has no effect on either the Na^+, K^+-ATPase, or the plasma membrane Ca^{2+}-ATPase families (Lytton *et al.*, 1991a; Sagara and Inesi, 1991; Campbell *et al.*, 1991). Despite these indications of similarity, it seemed likely that functional differences existed among the SERCA isoforms which, combined with tissue- or cell-specific expression, might impart unique properties of calcium homeostasis to certain cells. In order to test this hypothesis, we have used the COS cell expression system to analyze various biochemical parameters of the SERCA isoforms. Part of this work has been published previously in abstract form (Lytton *et al.*, 1991b).

EXPERIMENTAL PROCEDURES

Materials—COS-1 or COS-7 cells were obtained from several independent sources. We found that the quantity of SERCA enzyme expression varied dramatically depending upon the exact growth conditions and source of the cells. Our best results were obtained with a clonal derivative of COS-1, M6, isolated in Dr. Randal J. Kaufman's laboratory at Genetics Institute (Cambridge, MA). The SERCA cDNA constructs used for transfection were subcloned into the expression vectors p91023(B) or pRK1-4 (Genetics Institute) exactly as previously described (Lytton *et al.*, 1991a). Data concerning SERCA1 were obtained from the rabbit clone corresponding to the adult, "a" transcript. Previous work has established that SERCA1a and SERCA1b are functionally equivalent and, therefore, no further attempts were made to study SERCA1b. SERCA2a and SERCA2b data were obtained from either rabbit or human cDNA clones (pCA_3 and HK2, and RbUt2-1 and HK1, respectively), with identical results. SERCA3 data were from the rat clone RK8-13. Control constructs consisted of either vector alone or RK8-13 subcloned in the antisense orientation in p91023(B). The rabbit polyclonal antiserum C4, raised against a *trpE-SERCA2* gene fusion product (Lytton and MacLennan, 1988), was affinity purified by absorption to dog cardiac Ca^{2+}-ATPase immobilized on strips of nitrocellulose. After washing with 140 mM NaCl, 10 mM Tris-HCl, pH 7.5 (TBS), antibody was eluted in 0.1 M glycine, pH 2.7, neutralized with 1 M Tris-HCl, pH 9, and buffer exchanged into TBS using Sephadex G-25 columns. The quantity of antibody was estimated by absorption at 280 nm (1 mg/ml of rabbit IgG = 1.35 A_{280}) and then stabilized with 0.1% (final) bovine serum albumin, 0.01% thimerosal, and frozen in aliquots. Cell culture and immunological reagents were from GIBCO/BRL Life Technologies, Inc. 1.4-Diazabicyclo-[2,2,2]octane was purchased from EM Science. DEAE-dextran was from Pharmacia LKB Biotechnology Inc. [γ-^{32}P] ATP and $^{45}CaCl_2$ were purchased from Du Pont-New England Nuclear. Electrophoresis and electrophoretic transfer reagents and apparatus were from Bio-Rad. All other chemicals were obtained either from Sigma or from Fisher Scientific (molecular biology grade).

Cell Culture and Transfection—COS cells were maintained at 37 °C under 7% CO_2 in high glucose Dulbecco's modified Eagle's medium with pyruvate (DMEM) supplemented with L-glutamine to a total of 4 mM, 0.1 mM MEM non-essential amino acids, 10% fetal bovine serum, and 100 units/ml penicillin, 100 μg/ml streptomycin. DEAE-dextran-mediated transfection with chloroquine shock was accomplished essentially according to established protocols (Ausubel *et al.*, 1992; Maruyama and MacLennan, 1988; Clarke *et al.*, 1989b; Kaufman, 1990), with the following modifications. Cells at ~25–50% confluence in 10-cm dishes were washed twice with serum-free medium, then 1 ml of a mixture of 0.5 ml (10 μg/ml DNA in DMEM) and 0.5 ml (1 mg/ml DEAE-dextran in 140 mM NaCl, 5 mM KCl, 10 mM Tris-HCl, pH 7.5) was added to each dish. The dishes were incubated for 30 min in the CO_2 incubator, agitating gently at 15 min to prevent drying. The DNA solution was gently aspirated and replaced with 6 ml of fully supplemented medium containing 100 μM chloroquine. After 3 h, this solution was removed, the cells washed twice with DMEM, and grown for 48 h in fully supplemented medium.

Immunocytochemistry—One day following transfection (as above), cells were gently trypsinized and replated onto glass coverslips in 6-well dishes. The next day, the cells were washed twice with 130 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2 (PBS), fixed in acetone/methanol (1:1) at -20 °C for 2 min, then rinsed twice in PBS, and incubated at room temperature for 15 min in 3% (w/v) bovine serum albumin in PBS (BSA/TBS). The coverslips were then inverted over a 100-μl drop of affinity purified C4 antibody (2 μg/ml) in BSA/PBS on Parafilm and incubated in a sealed humidified dish for 1 h at room temperature, rinsed three times for 5 min each in PBS, inverted over a drop of fluorescein-conjugated goat-anti-rabbit IgG diluted in BSA/TBS, and incubated in the dark for 30 min. The coverslips were then rinsed again and mounted in 90% glycerol, 10 mM Tris-HCl, pH 8.5, containing 2.5% (w/v) 1,4-diazabicyclo-[2,2,2] octane (an anti-fade reagent). Immunofluorescence was observed with a Nikon Microphot FXA microscope using a band pass excitation filter (450–490 nm), a 510-nm chromatic beam splitter, a long pass barrier filter (520 nm) and a 100 × oil-immersion objective, and photographed on Kodak Ektachrome EES slide film, push-processed at an ASA setting of 800.

Membrane Preparations—A crude, post-mitochondrial membrane fraction, termed "microsomes" was obtained from the transfected COS cells as previously described (Maruyama and MacLennan, 1988; Clarke *et al.*, 1989b) and suspended at a final protein concentration of 0.5–2 mg/ml. Dog cardiac sarcoplasmic reticulum (prepared according to the method of Chamberlain and Fleischer (1988)) was a gift from J. Fujii (Osaka University, Japan). Rabbit fast-twitch skeletal muscle sarcoplasmic reticulum was prepared essentially as described by Campbell and MacLennan (1981) in the presence of 0.1 mM phenylmethylsulfonyl fluoride and 1 μM leupeptin. Protein concentrations were determined by the dye binding assay of Bradford (1976) using bovine γ-globulin as a standard with the Protein Assay kit from Bio-Rad.

Electrophoresis and Immunoblotting—Samples for immunoblotting were dissolved without heating in sample buffer containing 2% β-mercaptoethanol, resolved on 7.5% SDS-polyacrylamide gels according to Laemmli (1970), and electrophoretically transferred to nitrocellulose membranes (Towbin *et al.*, 1979). Detection using affinity purified C4 antibody (2 μg/ml) was as described (Lytton and MacLennan, 1988). For the detection of the phosphorylated enzyme-intermediate, 7% polyacrylamide gels were run according to the system of Weber and Osborn (1969), with the pH of the running buffer adjusted to 6.3. After electrophoresis, the gels were fixed for 15 min in 10% acetic acid, 5% methanol, dried, and exposed to x-ray film at −70 °C using an intensifying screen.

Assays—Calcium uptake, calcium-dependent ATP hydrolysis using the enzyme-coupled spectrophotometric assay system, and phosphorylated enzyme intermediate trapping were performed at a pH of 7.0 essentially as described previously (Lytton *et al.*, 1991a), except that the concentration of potassium oxalate in the uptake buffer was 5 mM. In general the reactions were initiated by the addition of membranes (5–10 μg of COS microsomes, 0.5–1 μg of fast-twitch skeletal muscle sarcoplasmic reticulum, or 1–5 μg of cardiac muscle sarcoplasmic reticulum), except for the experiments with vanadate, where membranes were preincubated with vanadate in buffer without ATP for 15 min at room temperature, and uptake was then initiated by the addition of ATP. In all the experiments except that of Fig. 1,

time points in the linear range were used (5–10 min for COS microsomes, 3–5 min for cardiac muscle sarcoplasmic reticulum, and 1–2 min for fast-twitch muscle sarcoplasmic reticulum). For the phosphorylated enzyme intermediate trapping experiments, the acid-precipitated protein was either dissolved in 200 μl of 0.1 M NaOH, 0.1% SDS, and the entire sample counted using Cherenkov radiation (for quantitation), or the pellet was washed quickly in ice-cold water, dissolved in 25 μl of sample buffer, and applied to an SDS-polyacrylamide gel run according to Weber and Osborn (1969) at pH 6.3 (see above).

Different concentrations of free calcium were generated using EGTA as a buffer. The EGTA-Ca^{2+} dissociation constant (K_{app}) was corrected for ionic strength (I) and pH according to the data of Harafuji and Ogawa (1980) using the following equation.

$$\log(K_{app}) = 6.46 - (2\sqrt{I}/(1 + \sqrt{I}) - 0.4\,I) + 2\,(pH - 6.8).$$

Although ATP also has the potential to complex calcium, under the conditions used for these experiments (micromolar free calcium, and the presence of (at least) an equimolar amount of magnesium as ATP), the contribution of ATP to calcium buffering is negligible and was ignored in the calculations. The free calcium concentration was adjusted either by maintaining the total calcium concentration at 0.5 mM (and hence the specific radioactivity of $^{45}CaCl_2$ in the uptake experiments remained constant) while adjusting the concentration of EGTA, or by varying calcium at a constant EGTA concentration of 0.5 mM, with identical results. For the pH dependence experiment, the above equation was used to calculate appropriate EGTA concentrations which maintained a free calcium concentration of 5 μM. In all other experiments where calcium was not varied, the free concentration was buffered to 3–5 μM with EGTA.

The data from experiments measuring the dependence of activity (v) upon either calcium or ATP concentration have been fit by computer to the general cooperative model for substrate (S) activation

$$v = V_{max}[S]^n/(K_{1/2}^n + [S]^n)$$

where V_{max} is the maximum activity reached (usually at maximum S), $K_{1/2}$ is the substrate concentration which gives half of V_{max}, and n is the equivalent to the Hill coefficient, which can be thought of simplistically as the number of substrate molecules required/turnover of the enzyme, recognizing that this is true only when substrate binding is a highly cooperative process (Inesi *et al.*, 1990). The program *SigmaPlot* (Jandel Scientific), run on an Apple Macintosh II computer, was used for general non-linear curve-fitting. The best fits for calcium gave an n value indistinguishable from 2, while those for ATP gave a value of 1.

RESULTS

Transient expression of the sarcoplasmic reticulum Ca^{2+}-ATPase in COS cells has proven to be very powerful system for addressing the relation of structure to function for this enzyme (Maruyama *et al.*, 1989). We have therefore chosen the same system to compare the biochemical properties of the different calcium pump isoforms. Fig. 1*A* shows an immunoblot, developed with affinity purified C4 polyclonal anti-Ca^{2+}-ATPase antibody, of microsomes isolated from COS cells transfected with each of the different SERCA constructs used in this paper. Each isoform was expressed at high levels compared to control transfected cells and migrated with a characteristic pattern. Interestingly, in our gel system SERCA1 migrated with an apparent size larger than predicted from its sequence compared with the other isoforms (SERCA1, 2a, and 3 are all predicted to be 109.5 kDa, while SERCA2b is 114.6 kDa). The SERCA2 gene products, including cardiac sarcoplasmic reticulum, all showed minor species running just below the major band. In addition, the SERCA2b protein demonstrated a susceptibility to aggregation, not seen with the other isoforms, suggesting that this might be related to the unique extended carboxyl-terminal sequence of this isoform.

The capacity for each of the expressed SERCA species to function as a calcium pump is illustrated in Fig. 1*B*, where

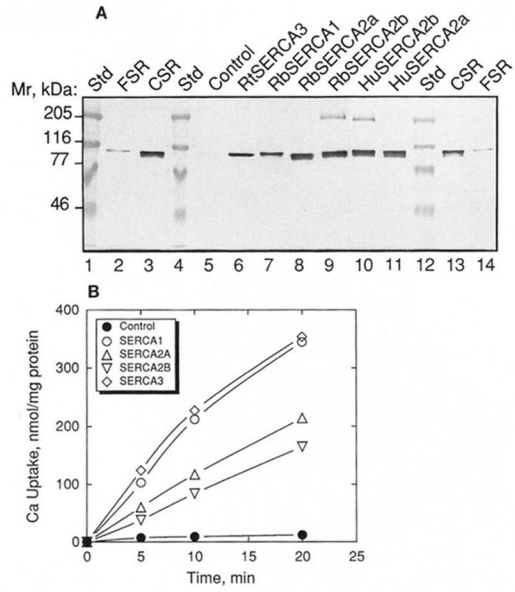

FIG. 1. **Expression of SERCA isoforms in COS cells.** *A*, microsomes (5 μg) from COS cells transfected with the indicated SERCA constructs were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with affinity purified C4 polyclonal anti-Ca^{2+}-ATPase antibody. *Hu*, human cDNA, *Rb*, rabbit cDNA, *Rt*, rat cDNA. *Std*, prestained molecular weight standards (high range) from Bio-Rad. In *lanes 1* and *4* these have not been reduced, and the third band from the top (*BSA*) runs with an aberrant mobility. The approximate size of the markers is indicated in the figure margin. *FSR*, sarcoplasmic reticulum membranes (0.1 μg) from rabbit fast-twitch skeletal muscle. *CSR*, sarcoplasmic reticulum membranes (0.5 μg) from dog cardiac muscle. Note that *lanes 1–9* and *lanes 10–14* are from different gels; the immunoreactive species at ~200 kDa seen in *lanes 9* and *10* comigrates if the samples are run together (data not shown). *B*, the time course of oxalate-dependent calcium uptake measured at room temperature into COS cell microsomes transfected with the indicated SERCA constructs. A typical experiment is shown; the rates of transport varied from one experiment to the next, ranging from 10 to 100 nmol/mg/min for SERCA1. See "Experimental Procedures" for further details.

oxalate-dependent calcium uptake into microsomes isolated from transfected COS cells is followed with time. It seemed possible that the differences in transport rate between SERCA isoforms reflected fundamentally different rates of turnover of the different enzymes. To address this issue we determined the quantity of active enzyme present in the transfected COS microsomes by trapping the covalent phosphorylated intermediate formed during enzyme turnover (E-P). Fig. 2*A* illustrates that, in the presence of 2 μM ATP, the calcium-dependent incorporation of radioactivity is restricted to the ~110-kDa band which represents the SERCA gene products in microsomes from transfected, but not from control cells. This specificity allowed us to quantify SERCA expression by following the amount of calcium-dependent acid-precipitable radioactivity formed from ATP of known specific radioactivity. Pilot experiments (not shown) established that 2 μM ATP and 15 s on ice produced maximal incorporation. E-P values of different COS microsome preparations confirmed that the variance in calcium uptake from preparation to preparation (for a single isoform) could be explained largely by different levels of active enzyme. Calcium uptake and calcium-dependent ATPase activity (both measured at 30 °C) of transfected COS microsomes, as well as sarcoplasmic reticulum from skeletal and cardiac muscle, were normalized to the amount of measured E-P, and the results are shown in Fig. 2*B*. While SERCA 1, 2a, and 3 displayed values not significantly different from those of either skeletal or cardiac sarcoplasmic

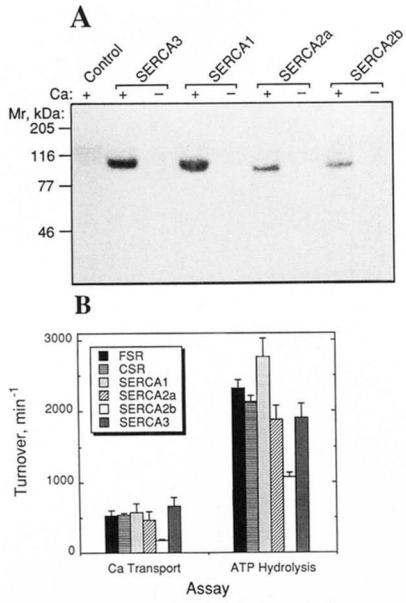

FIG. 2. **Phosphorylated intermediate formation and SERCA stoichiometry.** *A*, COS cell microsomes (5 μg) transfected with the indicated SERCA isoform constructs were phosphorylated for 15 s on ice either in the presence of 100 μM calcium, or in its absence (1 mM EGTA), precipitated with trichloroacetic acid, and separated on an SDS-polyacrylamide gel at pH 6.3 according to Weber and Osborn (1969). An autoradiogram of the fixed, dried gel is shown. The approximate size of the prestained molecular weight markers (Bio-Rad) is indicated in the *margin*. *B*, the enzyme turnover rates for oxalate-dependent calcium uptake and calcium-dependent ATP hydrolysis, both measured during the linear portion of the assays at 30 °C, are shown normalized to the content of calcium-dependent phosphorylated enzyme-intermediate (*E*-P) measured in each preparation. See "Experimental Procedures" for further details. *FSR*, sarcoplasmic reticulum membranes from rabbit fast-twitch skeletal muscle. *CSR*, sarcoplasmic reticulum membranes from dog cardiac muscle. The average ± standard error of the mean for duplicate determinations of turnover number on three different membrane preparations is shown. The average content of *E*-P was 2450, 470, 80, 45, 57, and 98 pmol/mg protein for FSR, CSR, SERCA1, SERCA2a, SERCA2b, and SERCA3, respectively.

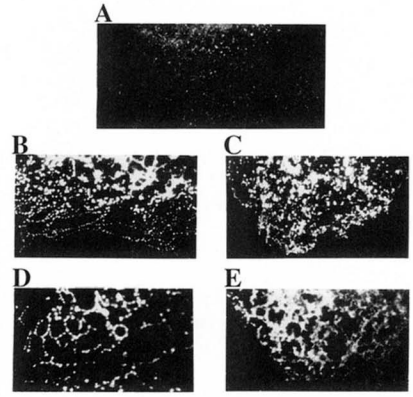

FIG. 3. **Immunofluorescence of SERCA expression in COS cells.** COS cells were transfected with control plasmid (*A*), or constructs encoding SERCA1 (*B*), SERCA2a (*C*), SERCA2b (*D*), or SERCA3 (*E*), and grown on glass coverslips. The cells were fixed and permeabilized in acetone/methanol at −20 °C and incubated with affinity purified C4 polyclonal anti-Ca^{2+}-ATPase antibody followed by fluorescein-conjugated secondary antibody. The periphery of selected cells is shown. See "Experimental Procedures" for further details.

reticulum Ca^{2+}-ATPase, SERCA2b had lower turnover rates for both calcium transport and ATP hydrolysis, suggesting a slower catalytic cycle for this isozyme. Our data are in contrast to the results of Campbell *et al.* (1991) who report equivalent rates of calcium uptake for SERCA1, 2a, and 2b. It is possible that their use of immunoblotting data to normalize uptake rates, rather than *E*-P measurements, provides the explanation for this discrepancy.

The sequence of SERCA3 contains a region at its carboxyl terminus which may encode a sorting signal not found in the other isoforms (Burk *et al.*, 1989). Furthermore, the alteration of SERCA2b activity might reflect a different membrane environment for that isoform. It was important, therefore, to examine the intracellular location of the SERCA gene products. Fig. 3 illustrates immunofluorescent micrographs of the flattened periphery of selected COS cells labeled using affinity purified C4 antibody followed by a fluorescein-conjugated secondary antibody. Routinely, 5–10% of the cells displayed a strong fluorescent signal, consistent with the expected efficiency of DEAE-dextran-mediated transfection (Ausubel *et al.*, 1992; Maruyama *et al.*, 1988). Fig. 3*A* shows the punctate fluorescent pattern visible in a typical cell from a control transfection. This pattern is indistinguishable from that of the faint cells in a SERCA construct transfection and presumably corresponds to endogenous SERCA expression in the endoplasmic reticulum of COS cells. *Panels B-E* of Fig. 3 are bright cells from SERCA1, 2a, 2b, and 3 transfections, respectively. A typical reticular pattern is observed, with some punctate concentration, suggesting that all SERCA isoforms are expressed in the same, or a similar, membrane compartment with a morphology resembling the endoplasmic reticulum. SERCA3 displayed the most diffuse reticular, least punctate, pattern of all the different isoforms.

A critical parameter for the biological functioning of calcium pumps is their affinity for intracellular calcium, which is also an important point of allosteric regulation both in cardiac muscle sarcoplasmic reticulum and for the plasma membrane Ca^{2+}-ATPase (Lytton and MacLennan, 1991; Carafoli, 1992). Calcium affinity was investigated by monitoring the calcium dependence of oxalate-dependent calcium uptake. Fig. 4 shows that COS cell microsomes expressing SERCA1 displayed a dependence virtually identical to that of fast-twitch skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase, with a $K_{1/2}$ of ~0.4 μM. SERCA2a expressed in COS cells also had a $K_{1/2}$ of ~0.4 μM, contrasting with its apparent affinity in cardiac muscle sarcoplasmic reticulum ($K_{1/2}$ about 0.9 μM) where the enzyme is known to be reversibly modulated through an association with the inhibitory protein, phospholamban (Tada *et al.*, 1988; Fujii *et al.*, 1990; Sasaki *et al.*, 1992). Although SERCA2b seemed to display a slightly higher apparent affinity for calcium ($K_{1/2}$ of 0.27 μM) compared to the other isoforms, this difference may not be significant because the low rate of uptake for SERCA2b (see Figs 1*B* and 2*B*) introduces greater uncertainty in the data points at low calcium concentration. The $K_{1/2}$ values we observe for SERCA 1, 2a, and 2b are consistent with those obtained by others previously (Fujii *et al.*, 1990; Campbell *et al.*, 1991). SERCA 3, on the other hand, had a much lower apparent calcium affinity, $K_{1/2}$ about 1.1 μM, very similar to that observed for the Ca^{2+}-ATPase of cardiac sarcoplasmic reticulum. The difference in affinity between SERCA1 and 3 was confirmed using two independent assays: Ca^{2+}-ATPase activity and *E*-P formation, as shown in Fig. 5. In all cases, the data for calcium dependence of enzyme activity were best fit by a model requiring two highly cooperative calcium-binding sites for activity (*i.e.* a Hill coefficient of about 2).

One of the regions of largest sequence divergence among the SERCA isoforms is in the nucleotide-binding domain. Thus, it seemed plausible that differences in ATP activation

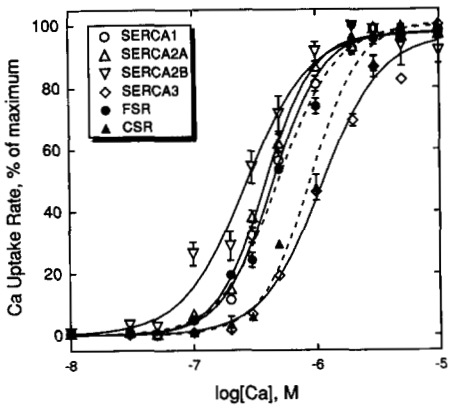

FIG. 4. **Calcium dependence of calcium transport activity.** The calcium dependence of the relative rate of calcium uptake is shown for microsomes from COS cells transfected with the indicated SERCA constructs. For comparison, the data obtained using sarcoplasmic reticulum from either rabbit fast-twitch skeletal muscle (*FSR*) or dog cardiac muscle (*CSR*) are also plotted (*dashed curves*). Measurements were at room temperature during the linear portion of uptake. The averages from between three and 17 independent determinations are shown. The *curves* represent the best fit of the data to a cooperative model of enzyme activation obtained by general non-linear computer-assisted curve-fitting. See "Experimental Procedures" for further details. The n and $K_{1/2}$ values ± the standard deviation for SERCA1, SERCA2a, SERCA2b, SERCA3, FSR, and CSR, respectively, are: 2.1 ± 0.1, 0.44 ± 0.01 μM; 2.2 ± 0.1, 0.38 ± 0.01 μM; 1.7 ± 0.3, 0.27 ± 0.03 μM; 1.8 ± 0.2, 1.1 ± 0.1 μM; 1.8 ± 0.2, 0.49 ± 0.03 μM; 2.1 ± 0.4, 0.9 ± 0.1 μM.

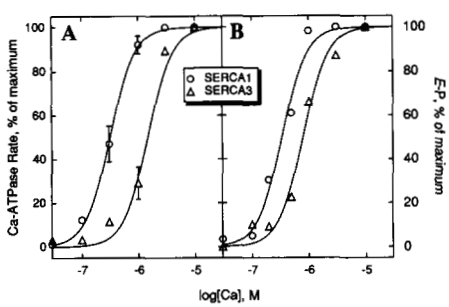

FIG. 5. **Calcium dependence of Ca^{2+}-ATPase and *E*-P formation.** Microsomes from COS cells transfected with SERCA1 or SERCA3 demonstrate different apparent calcium affinities as measured by the calcium dependence of either Ca^{2+}-ATPase activity (*panel A*) or phosphorylated enzyme-intermediate formation, *E*-P (*panel B*). The averages of two or three separate determinations are shown. The *curves* represent the best fit of the data to a general cooperative model with n set to 2. See "Experimental Procedures" for further details. The $K_{1/2}$ values for *panels A* and *B*, respectively, are: SERCA1, 0.31 and 0.34 μM; SERCA3, 1.4 and 0.8 μM.

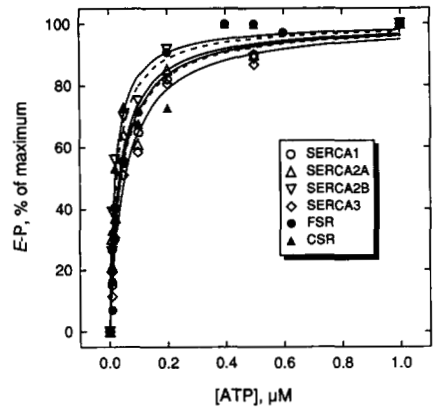

FIG. 6. **ATP dependence of *E*-P formation.** The ATP dependence of phosphorylated enzyme-intermediate (*E*-P) formation is shown for microsomes from COS cells transfected with the indicated SERCA constructs. For comparison, the data obtained using sarcoplasmic reticulum from either rabbit fast-twitch skeletal muscle (*FSR*) or dog cardiac muscle (*CSR*) are also plotted (*dashed curves*). The averages of duplicate determinations are shown. The *curves* represent the best fit of the data to a simple one-site model of enzyme activation obtained by general non-linear computer-assisted curve-fitting. See "Experimental Procedures" for further details. The $K_{1/2}$ values for SERCA1, SERCA2a, SERCA2b, SERCA3, FSR, and CSR, respectively, are (in μM): 0.04, 0.04, 0.02, 0.05, 0.04, and 0.03.

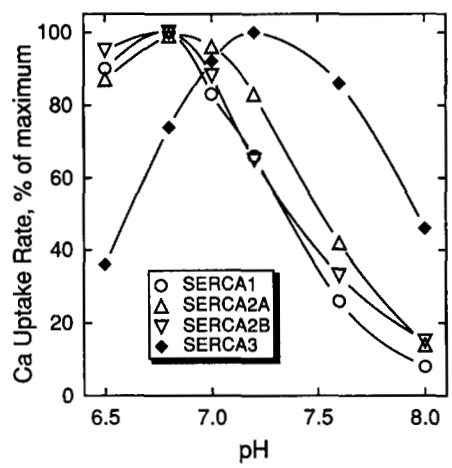

FIG. 7. **pH dependence of calcium transport.** The rate of calcium transport during the linear portion of the uptake reaction was determined at different pH values for microsomes isolated from COS cells transfected with the indicated SERCA constructs. See "Experimental Procedures" for further details.

or binding properties might exist among the isoforms. ATP binds to the enzyme with high affinity at the catalytic site, donating the γ-phosphate to the active site aspartate residue during turnover of the enzyme. The high affinity catalytic ATP-binding site was investigated by following the ATP dependence of *E*-P formation, as illustrated in Fig. 6. All of the microsomes from transfected COS cells as well as sarcoplasmic reticulum preparations from skeletal and cardiac muscle displayed virtually identical dependencies with $K_{1/2}$ values ranging from 0.02–0.05 μM, and no sign of cooperativity (*i.e.* $n = 1$). The dependence of the overall enzyme reaction upon ATP, particularly at higher concentrations which occupy a low affinity regulatory site (Inesi, 1985; Inesi and de Meis, 1985), was not determined.

Carboxylate residues have been implicated in the high affinity calcium-binding sites involved in transport (Clarke *et al.*, 1989a). Furthermore, it has been suggested that the sarcoplasmic reticulum Ca^{2+}-ATPase may transport protons in the counter direction to calcium (Levy *et al.*, 1990). pH, therefore, appears to be an important parameter of enzyme function, and its influence on enzyme activity was examined, as shown in Fig. 7. COS cell microsomes expressing SERCA1, 2a, or 2b displayed essentially superimposable curves, which matched the accepted pH dependence of the sarcoplasmic reticulum Ca^{2+}-ATPase: an optimum at about pH 6.8–7.0, which falls off rapidly at higher values (Inesi and de Meis, 1985). In contrast, the optimum pH for SERCA 3 was shifted to about pH 7.2–7.4, with activity dropping off sharply below pH 7.0. Note, that at the pH of 7.0 used for all experiments, except those of Fig. 7, all isoforms are functioning close to their maximal rates.

Vanadate is a universal inhibitor of enzymes which form phosphorylated intermediates during their reaction cycle, probably because it resembles the transition state of the γ-

phosphate of ATP during transfer to enzyme, and thus binds at the active site (Cantley *et al.*, 1978). In addition, it has been reported that certain cells possess an ATP-dependent calcium uptake into subcellular organelles which is resistant to vanadate (Thevenod *et al.*, 1989). Therefore, we investigated the interaction of vanadate with the SERCA isoforms by measuring the dose-response for vanadate inhibition of calcium uptake. The results, shown in Fig. 8, establish that SERCA3 displays a markedly higher apparent affinity for vanadate than the other SERCA isoforms. This is consistent with the mutually competitive effects of calcium and vanadate on the Ca^{2+}-ATPase (Inesi, 1985).

DISCUSSION

We have employed a COS-1 cell expression system to examine the properties of different SERCA isoforms and found that the two non-muscle SERCA pumps, while similar in many respects to their muscle counterparts, also displayed clear functional differences. Immunocytochemical analysis revealed that all of the isoforms were present within the same, or similar, compartment when expressed in COS cells, as has been reported for chicken SERCA1, 2a, and 2b (Campbell *et al.*, 1991). This is consistent with their similar behavior during cell fractionation and their similar calcium transport properties (such as oxalate dependence), both of which would be expected to change if the isoforms resided in distinct compartments. Accordingly, the COS-1 cell system appears to sort this group of proteins appropriately.

The high degree of sequence similarity among all of the SERCA isoforms, particularly the identity of residues implicated by mutagenesis studies as critical for enzyme function, suggests that these isozymes share a fundamental mechanism for binding calcium and coupling the subsequent hydrolysis of ATP to active transport (MacLennan, 1990; Inesi *et al.*, 1990). Our finding of qualitatively similar transport capacities, similar calcium and ATP affinities, and most notably, virtually identical Hill coefficients supports this notion. Nevertheless, we did observe that SERCA2b turns over significantly more slowly than the other isoforms and that SERCA3 has a reduced affinity for calcium, an increased affinity for the inhibitor vanadate, and an altered pH dependence when compared to its family members.

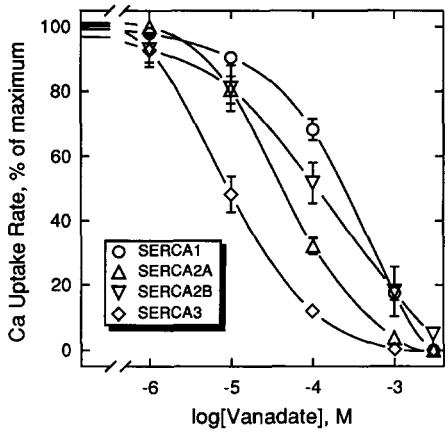

FIG. 8. **Vanadate inhibition of calcium transport.** Microsomes from COS cells transfected with the indicated SERCA constructs were preincubated in uptake buffer without ATP in the presence of different vanadate concentrations for 15 min at room temperature. Calcium uptake was initiated by the addition of ATP and stopped 10 min later. The data, which represent the average ± standard error of the mean for between three and nine separate determinations, are plotted as a percentage of the activity without vanadate.

The calculations for turnover of the enzyme are critically dependent upon the estimate of *E*-P corresponding quantitatively to the amount of active enzyme. We have established that incorporation of radioactivity is maximal and constant under the conditions employed here (data not shown). Importantly, the absence of ionophore allows calcium to accumulate within the vesicles, inhibiting calcium release and subsequent *E*-P hydrolysis. These conditions have been demonstrated to result in stoichiometric trapping of phosphate on the Ca^{2+}-ATPase (Barrabin *et al.*, 1984). Furthermore, a qualitative estimate of the relative levels of SERCA2a to SERCA2b expression can be determined by examining the C4 antibody immunoblots (Fig. 1*A*). This confirms the *E*-P data demonstrating that SERCA2b is expressed at a level similar to SERCA2a.

We believe that the reduced activity of SERCA2b reflects a lower enzyme turnover rather than a different number of calcium ions transported/reaction cycle for two reasons. First, the calcium dependence of activity displays cooperativity with a Hill coefficient close to 2. Second, measurements of turnover for ATP hydrolysis and measurements of turnover for calcium transport are both reduced for SERCA2b (46 and 33% of the fast-twitch muscle sarcoplasmic reticulum values, respectively). There is more scatter, however, in the values for ATP hydrolysis than in the values for calcium uptake. The low level of Ca^{2+}-ATPase expression in COS microsomes compared to sarcoplasmic reticulum precludes the rapid kinetic analysis required to measure true ratios between calcium transport and ATP hydrolysis (Inesi, 1985). Under the conditions necessary to measure calcium transport in COS microsomes (particularly the presence of 5 mM oxalate), the activity of the Ca^{2+}-ATPase is dramatically reduced, and thus the ratio of calcium transported to ATP hydrolyzed is much lower than the theoretical value of 2 (Ca^{2+}/ATP ratios measured under our conditions were in the range of 0.2–0.4 for both COS microsomes and sarcoplasmic reticulum preparations). It is intriguing to speculate that the extended SERCA2b carboxyl terminus may allosterically control enzyme activity, as has been shown for the carboxyl calmodulin-binding domain of the plasma membrane Ca^{2+}-ATPase (Carafoli, 1992).

The apparent calcium affinities of expressed SERCA1, 2a, and probably 2b, are all similar (as also shown by Campbell *et al.* (1991) for the chicken isoforms) and identical to that measured for the Ca^{2+}-ATPase of sarcoplasmic reticulum from fast-twitch skeletal muscle (which expresses SERCA1a). Cardiac muscle sarcoplasmic reticulum Ca^{2+}-ATPase, on the other hand, displays a lower apparent affinity, despite expressing SERCA2a. It has been established that this results from the interaction of the Ca^{2+}-ATPase with the integral membrane protein phospholamban in cardiac muscle sarcoplasmic reticulum (Tada *et al.*, 1988; Fujii *et al.*, 1990; Sasaki *et al.*, 1992). Thus, the properties of the SERCA isoforms which we have determined in COS cells may not necessarily correspond to those of the molecules expressed in their native environment, where additional modulators may also be present.

The reduced affinity of SERCA3 for calcium compared to the other isoforms was somewhat unexpected, since all the residues implicated by mutagenesis studies to be involved in complexing calcium, or in positioning the binding ligands, are conserved (MacLennan, 1990; Lytton and MacLennan, 1991). An interesting comparison can be drawn between SERCA3 and the interaction of the Ca^{2+}-ATPase with phospholamban, which also results in a reduction of the apparent calcium

affinity of the enzyme. Recently, Sasaki *et al.* (1992) reported that a truncated, largely hydrophobic, phospholamban, missing the cytosolic amino terminus, retained the ability to shift the calcium affinity of the enzyme. A possible interpretation of these data is that an interaction between hydrophobic helices within the bilayer influences the position of key calcium-complexing residues, hence altering the binding affinity. The extension of this result is that amino acid differences within the bilayer between SERCA3 and other isoforms, although not close to the calcium-binding site, might nevertheless influence calcium affinity through helix-helix interactions.

There is an alternative explanation for calcium affinity shifts though, which results from the equilibrium between two major conformations of the Ca^{2+}-ATPase, E_1 and E_2. Cytoplasmic calcium binds with high affinity to E_1 and not to E_2. If unoccupied E_1 and E_2 are in dynamic equilibrium, then shifting that equilibrium one way or the other will influence the apparent calcium affinity. In this model, phospholamban is thought to interact with the enzyme to shift the equilibrium toward E_2, hence lowering the apparent calcium affinity (Tada *et al.*, 1988). Similarly, it is possible that the reduced calcium affinity of SERCA3 reflects an intrinsic alteration in the E_1 to E_2 equilibrium. The vanadate inhibition data are consistent with such a model. Vanadate binds to the E_2 conformation of the enzyme, and thus the apparent vanadate affinity also depends upon the E_1 to E_2 equilibrium, but in an inverse manner to calcium which binds to E_1. Thus, a shift toward E_2, which will result in lower apparent calcium affinity, is expected to raise the apparent vanadate affinity. As seen in Fig. 8, SERCA3 is inhibited by almost 10-fold lower concentrations than the other SERCA isoforms, and thus has an apparently higher vanadate affinity.

The alkaline shift in the pH optimum for SERCA3 is consistent with a model of H^+ competition at the high affinity calcium-binding sites (Inesi and de Meis, 1985). If, for example, carboxylate residue(s) participate in complexing calcium, then a rise in the pK of that residue would lead to a reduced calcium affinity. It is also possible, however, that the pH shift has its basis in the E_1 to E_2 equilibrium, just as the effects on both calcium and vanadate affinities may. This hypothesis is appealing because a single mechanistic alteration can explain all three experimental observations.

The expression of SERCA isoforms in COS cells has revealed clear functional differences between the non-muscle isoforms (SERCA2b and 3), and their muscle counterparts (SERCA1 and 2a). It is anticipated that a knowledge of the underlying structural alterations will provide new insights into the function of this important class of enzymes. The reduced turnover rate of SERCA2b suggests the possibility for an interaction of its extended carboxyl-terminal tail with another part of the molecule. The reduced calcium affinity of the SERCA3 isoform is especially intriguing, since at normal resting intracellular calcium levels ($\leq 10^{-7}$ M) it would be essentially inactive. This raises the possibility that the calcium affinity of SERCA3 is allosterically modulated *in situ* or that this isoform is expressed in an environment of chronically elevated calcium. The different pH and calcium dependencies of SERCA3 activity, as well as the similarity of its carboxyl terminus with the hypothesized endoplasmic reticulum retention signal of the adenovirus E3/19k protein, suggest that this isoform might be targeted to a unique subcellular location. At the low level of resolution of the micrographs shown in Fig. 3, we were unable to detect any specific subcellular localization of SERCA3 in COS cells. Examination of tissue mRNA expression has revealed a high level of SERCA3 expression in colon and cerebellum (Burk *et al.*, 1989).[2] It will be interesting to determine which cell type expresses SERCA3, and whether or not there is a unique subcellular distribution of the enzyme, in these tissues.

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