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J. M. O'Donnell, A. Fields, X. Xu, S. A. K. Chowdhury, D. L. Geenen and J. Bi

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G. Smith

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Expression of SERCA isoform with faster Ca²⁺ transport properties improves postischemic cardiac function and Ca²⁺ handling and decreases myocardial infarction

M. A. H. Talukder, A. Kalyanasundaram, X. Zhao, L. Zuo, P. Bhupathy, G. J. Babu, A. J.

Cardounel, M. Periasamy and J. L. Zweier

Am J Physiol Heart Circ Physiol, October 1, 2007; 293 (4): H2418-H2428.

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Phenylephrine hypertrophy, Ca²⁺-ATPase (SERCA2), and Ca²⁺ signaling in neonatal rat cardiac myocytes

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Am J Physiol Cell Physiol, June 1, 2007; 292 (6): C2269-C2275.

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Comparison of SERCA1 and SERCA2a expressed in COS-1 cells and cardiac myocytes

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Sumbilla, Carlota, Marco Cavagna, Lilin Zhong, Hailun Ma, David Lewis, Iain Farrance, and Giuseppe Inesi. Comparison of SERCA1 and SERCA2a expressed in COS-1 cells and cardiac myocytes. *Am. J. Physiol.* 277 (*Heart Circ. Physiol.* 46): H2381–H2391, 1999.—Cultured COS-1 cells, as well as chicken embryonic and neonatal rat cardiac myocytes, were infected with recombinant adenovirus vectors to define limiting factors in the expression and Ca^{2+} transport function of recombinant sarcoplasmic-endoplasmic reticulum Ca^{2+} (SERCA) isoforms. Titration experiments showed that all COS-1 cells and myocytes in culture could be infected by an adenovirus titer of 10 plaque-forming units (pfu) per seeded cell. Raising the adenovirus titer further yielded higher protein expression up to an asymptotic limit for functional, membrane-bound SERCA protein. The asymptotic behavior of SERCA expression was not transcription related but was due to posttranscriptional events. The minimal (–268) cardiac troponin T (cTnT) promoter was a convenient size for adenovirus vector construction and manifested tight muscle specificity. However, its efficiency was lower than that of the nonspecific cytomegalovirus (CMV) promoter. At any rate, identical maximal levels of SERCA expression were obtained with the CMV and the cTnT promoter, as long as the viral titer was adjusted to compensate for transcription efficiency. A maximal threefold increase of total SERCA protein expression over the level of the endogenous SERCA of control myocytes was reached (a sevenfold increase compared with the endogenous SERCA of the same infected myocytes due to reduction of endogenous SERCA after infection). In contrast with previous reports [Ji et al. *Am. J. Physiol.* 276 (*Heart Circ. Physiol.* 45): H89–H97, 1999], a higher kinetic turnover was demonstrated for the SERCA1 compared with the SERCA2a isoform as shown by a 5.0- versus 2.6-fold increase in calcium uptake rate accompanying maximal expression of recombinant SERCA1 or SERCA2a, respectively. This information is deemed necessary for studies attempting to modify myocardial cell function by manipulation of SERCA expression.

calcium adenosine 5'-triphosphatase; sarcoplasmic-endoplasmic reticulum calcium; adenovirus vectors

THE SARCOPLASMIC-ENDOPLASMIC RETICULUM CALCIUM (SERCA) ATPase plays an important role in storing intracellular Ca^{2+} and regulating cytosolic Ca^{2+} concentration. A number of cell functions, including muscle contraction and relaxation, are thereby dependent on the activity of this enzyme in situ. Furthermore, defects of SERCA expression and intracellular Ca^{2+} homeosta-

sis appear to be associated with pathological states of cardiac muscle (12, 26, 28).

Various SERCA isoforms and gene-splicing products have been cloned (2, 23), including SERCA1, which is the isoform expressed specifically in skeletal muscle (2), and SERCA2a, which is prevalently expressed in cardiac muscle (35). Subsequently, SERCA cDNA constructs have been used for heterologous expression in COS-1 cells and in cardiac muscle to perform mutational analysis of recombinant enzyme (25) or used to study the influence of SERCA overexpression on cell functions (7, 9, 10, 13, 15, 16, 22). It is not yet clear, however, whether the yield of heterologous SERCA in cardiac myocytes is subjected to limits of transfection efficiency, transcription, translation, and functional folding of the expressed protein. Furthermore, the efficiencies and cell specificities of various promoters in cardiac myocytes are not yet defined. Finally, a detailed characterization of the two isoforms with regard to heterologous expression and comparative functional behavior is not available. Yet, all of this information is necessary if attempts are to be made to influence Ca^{2+} homeostasis and related functions by manipulation of SERCA expression in cardiac myocytes.

We describe in this paper a series of studies on heterologous expression of SERCA1 and SERCA2a isoforms in COS-1 cells and in chicken embryonic and neonatal rat cardiac myocytes, providing a systematic characterization of gene transfer procedures, effects of promoters, limits of protein expression, and comparative features of the recombinant SERCA1 and SERCA2a isoforms.

MATERIALS AND METHODS

DNA constructs and vectors. Enhanced green fluorescence protein (EGFP) and chicken (17) or rabbit SERCA1 (23) or SERCA2a (4, 35) cDNA were subcloned into the shuttle plasmid p dE1sp1A (Microbix Systems), preceded by various promoters and followed by SV40 polyadenylation signal. Shuttle plasmids were used for transfection of HEK-293 cells in conjunction with the replication-defective viral DNA plasmid pJM17 (Microbix Systems) to obtain recombinant adenovirus (8).

Alternatively, analogous shuttles were constructed utilizing the plasmid pAdlox. These shuttle plasmids were used for transfection of CRE8 cells in conjunction with the replication-defective adenovirus psi5, to obtain recombinant adenovirus vectors according to the methods of Hardy et al. (11).

In either case, the shuttle plasmids were constructed such that homologous recombination resulted in antisense direction of the gene of interest with respect to the adenovirus E1 gene promoter. The recombinant products were selected by repetitive replications in HEK-293 or CRE8 cells and band

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purified by centrifugation in cesium chloride gradients to yield concentrations of the order of 10⁶–10⁷ plaque-forming units (pfu)/ μ l.

Cell cultures. Cultures of HEK-293 cells, CRE8 cells, and COS-1 cells were maintained as described by Graham and Prevec (8), Hardy et al. (11), and Sumbilla et al. (31), respectively.

Primary cell cultures of cardiac myocytes and skin fibroblasts were obtained from *day 8* chicken embryos as previously described (15). Rat neonatal cardiac myocyte cultures were prepared as described in Kariya et al. (18).

Infections of cultured cells with recombinant adenovirus vectors were performed with 0.5–20 pfu/seeded cell in serum-free medium. Ninety minutes thereafter, the infection medium was diluted fourfold with culture medium containing 100 U of Pen-Strep and 1 μ g of Fungizone/ml. Two days after the infection the cells were harvested for microscopic or biochemical tests.

The shuttle plasmids described in *DNA constructs and vectors* were also utilized as such (without adenovirus recombination) for transfection of cultured cells. In this case transfections were performed by the DEAE-dextran method as described by Sumbilla et al. (31), by utilization of the liposome PerFect kit (Invitrogen), or by the calcium phosphate method described in Kariya et al. (18).

In situ evidence of heterologous expression was obtained by fluorescence microscopy of cell lawns simply rinsed with phosphate-buffered saline (PBS) (in the case of EGFP) or following staining with specific monoclonal antibodies and secondary fluorescent antibodies (in the case of the SERCA isoforms) as described by Inesi et al. (15).

Cell harvesting and protein analysis. Cell lawns were rinsed with cold PBS, and a cold suspension medium (10 ml per 100-mm dish) containing 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.0, 10 mM NaF, 1 mM EDTA, 0.3 M sucrose, 0.5 mM dithiothreitol, 0.4 mM Pefabloc, 10 μ g/ml aprotinin, 1 μ g/ml pepstatin A, and 2 μ g/ml leupeptin was added. The cells were collected by scraping with a spatula, and the resulting suspension was centrifuged at 2,000 *g* for 2 min. The sedimented cells were then resuspended in 1 ml of the same medium, frozen in liquid nitrogen, and stored at –70°C. Within 2 wk of storage the frozen cells were thawed and homogenized with 100 strokes of a hand-held homogenizer immediately before use for functional tests (see *Functional studies*). In some cases, COS-1 cells were homogenized and subjected to differential centrifugation for preparation of microsomes as described by Strock et al. (30).

Total protein was determined either by direct reading of 280-nm light absorbance following solubilization in 1% sodium dodecyl sulfate or by the bicinchoninic acid assay (Pierce) following sonication. Protein electrophoretic analysis was performed in 7.5% acrylamide gels according to Laemmli (20) and staining with Coomassie Blue. For Western blots the proteins were transferred onto nitrocellulose paper and stained with primary monoclonal and secondary (goat anti-mouse IgG-horseradish peroxidase conjugated) antibodies. The monoclonal antibodies (Affinity Bioreagents) were either specific for SERCA1 or SERCA2a protein or reactive to a common *c-myc* tag linked to the COOH terminal of both isoforms. Monoclonal antibodies were also used for detection of calsequestrin and phospholamban. The reactive bands were visualized using an enhanced chemiluminescence-linked detection system (Amersham Pharmacia Biotech), and their density was quantitated with the aid of a NucleoTech work station and Gel Expert software.

Northern blots. Total RNA was extracted by aspirating the medium from cell culture dishes, adding 0.3–0.4 ml of TRIzol

LS (GIBCO-BRL) per 10 cm² cell lawn, and following the GIBCO-BRL procedure. The final RNA pellet was dissolved in diethyl pyrocarbonate-water (Quality Biological) to yield ~1 mg RNA/ml. The final yield was 50 μ g of total RNA from one 150-mm dish.

Electrophoresis and blotting were performed by the use of the Northern Max-Gly Blotting Kit from Ambion. mRNA detection was obtained with a DIG High Prime Labeling and Detection kit from Boehringer Mannheim. SERCA1 cDNA was used as a template for the probe.

Functional studies. ATP-dependent Ca²⁺ transport was measured in a reaction mixture containing 40 mM MOPS (pH 7.0), 100 mM KCl, 5.0 mM MgCl₂, 5.0 mM NaN₃, 0.2 mM EGTA, 1 μ M ruthenium red, 0.2 mM [⁴⁵Ca]CaCl₂, and cell lysate (30–60 μ g/ml) or microsomal (5–10 μ g/ml) protein. The reaction was started by the addition of 5.0 mM potassium oxalate, and after 2 min, 5.0 mM ATP was added at 37°C. Samples were collected before and, at serial times, after the addition of ATP. The samples (1 ml each) were passed through 0.45- μ m Millipore filters, which were then washed with 2.0 mM LaCl₃ in 10 mM MOPS (pH 7.0), blotted, and used for determination of radioactive calcium by scintillation counting.

ATPase activity was measured in a reaction mixture containing 20 mM MOPS (pH 7.0), 80 mM KCl, 3.0 mM MgCl₂, 5.0 mM NaN₃, 0.2 mM EGTA, 0.2 mM CaCl₂, 3 μ M calcium ionophore A-23187, and 30–100 μ g/ml cell lysate or microsomal protein. The reaction was started by the addition of 3.0 mM ATP at 37°C. Serial samples were collected before and after addition of ATP. P_i was determined according to Lanzetta et al. (21).

Phosphoenzyme decay rates were determined by first obtaining steady-state levels of radioactive phosphorylated enzyme intermediate by adding 0.1 ml of 10 μ M [³²P]ATP to 0.4 ml of a reaction mixture containing 20 mM MOPS (pH 7.0), 80 mM KCl, 5.0 mM MgCl₂, 0.1 mM CaCl₂, and 30–100 μ g of microsomal protein. The components of the reaction mixture were preincubated in ice, and vortex mixing was carried out in the cold room. Ten seconds after the addition of radioactive ATP, a pulse chase of 0.5 ml of 1.0 mM nonradioactive ATP was added with rapid mixing, and samples were quenched at various times by adding 0.4 ml of 4 M perchloric acid. Two zero-time samples were obtained by quenching before the addition of nonradioactive ATP. The quenched samples were transferred onto Eppendorf tubes containing 100 μ g of carrier protein and centrifuged at 5,000 rpm for 5–10 min. The sediment was washed twice with 0.125 M perchloric acid and once with water. The entire procedure was carried out in ice and in the cold room. Aliquots of the solubilized samples were subjected to electrophoresis (34) at pH 6.3, and the radioactive phosphoenzyme was detected by autoradiography and phosphoimaging.

RESULTS

Efficiency of gene transfer in cultured cells. It is known that expression in COS-1 cells is enhanced if the exogenous gene is placed under control of the SV40 promoter. This enhancement is due to amplification of the transfected plasmid, triggered by SV40 promoter interaction with T-antigen in the COS-1 cells. The overall protein expression in cell cultures, however, is limited drastically by the small percentage (2–5%) of cells effectively transfected by procedures based on the use of calcium phosphate, DEAE-dextran, or liposomes. This limit can be overcome by the use of recombinant adenovirus vectors, which allow effective transfection

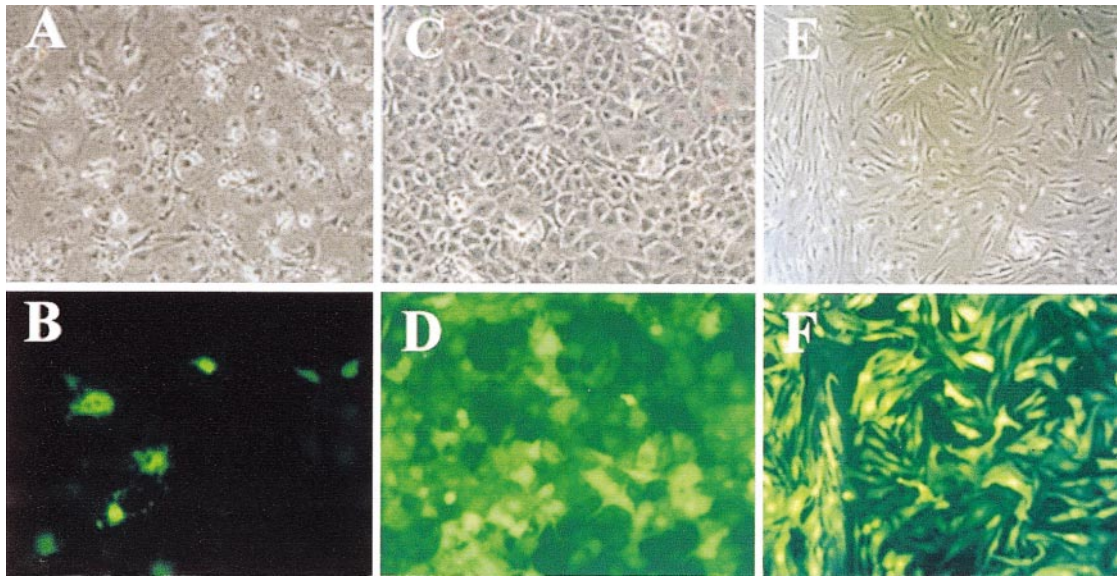


Fig. 1. Phase and fluorescent photomicrographs comparing DEAE-dextran transfection (*A* and *B*) and recombinant adenovirus infection (*C-F*) efficiencies in COS-1 cells (*A-D*) and chicken embryonic cardiac myocytes (*E* and *F*). In pairs *A* and *B*, *C* and *D*, and *E* and *F*, top panel shows phase contrast image, whereas bottom panel shows corresponding fluorescent cells, indicating percentage of cells expressing enhanced green fluorescence protein (EGFP) gene in same samples. COS-1 cells were transfected using a DEAE-dextran method as previously described (31). COS-1 cells or myocytes were infected with 10 plaque-forming units (pfu)/cell of a recombinant adenovirus expressing EGFP. EGFP cDNA was under control of cytomegalovirus (CMV) promoter in both transfection plasmid and recombinant adenovirus vector. Two days after transfection or infection, cells were examined using a Zeiss Axioscope with a $\times 10$ objective, an HB 100-W mercury lamp, and fluorescence attachments.

of nearly 100% of the COS-1 cells in culture. We found, however, that complementation of the SV40 promoter with the COS-1 cell T antigen does not trigger amplification of the recombinant gene caused by limits imposed by the viral DNA. Therefore, heterologous protein expression is highly dependent on the strength of the promoter. For these reasons the highest overall level of expression in cell cultures is obtained by placing the cDNA under control of the cytomegalovirus (CMV) promoter and using recombinant adenovirus vectors. Thereby, high percentages of transfected cells, as well as high levels of expression in each cell, are obtained in both COS-1 cells and chicken embryonic cardiac myocytes (Fig. 1). High expression levels were also obtained with neonatal rat cardiac myocytes (results not shown).

In experiments with adenovirus vectors, an important variable is the number of viral particles (pfu) per infected cell. We defined this variable in our experiments by exposing either COS-1 cells or cardiac myocytes to increasing titers of recombinant adenovirus vector carrying the EGFP DNA-coding sequence under control of the CMV promoter. In these experiments the percentage of cells expressing EGFP was conveniently determined by simply comparing phase and fluorescent microscopic views of the cells in culture. It should be pointed out that determinations of plaque-forming units per cell ratios are approximate, because their accuracy is limited by various uncertainties, including the degree of cell proliferation within the time interval (i.e., 2 days) intervening between cell infections and harvesting. It can be safely concluded, however, that nearly 100% of both COS-1 cells and myocytes produce EGFP following exposure to 10–20 pfu per seeded cell (Fig. 2).

Similar results were obtained when the adenovirus vector carried the SERCA DNA-coding sequence and SERCA ATPase expression was visualized by immunostaining and fluorescence microscopy (15).

Overall expression of EGFP and SERCA ATPase. We found that overall protein expression was further increased when the plaque-forming unit level was raised above levels affecting 100% of the cells in culture (i.e., 10–20 pfu/cell). In fact, in experiments with either COS-1 cells or myocytes, EGFP expression was linearly related to the viral titer (Fig. 3), demonstrating depen-

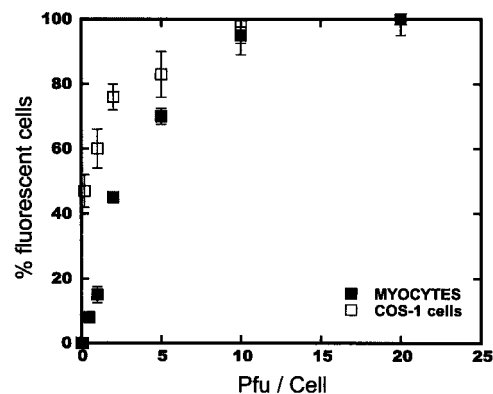


Fig. 2. Percentage of COS-1 cells or chicken embryonic cardiac myocytes expressing EGFP after infection with various plaque-forming unit levels of adenovirus vector. Cells were infected with increasing plaque-forming unit levels of EGFP adenovirus vector as described in MATERIALS AND METHODS. EGFP cDNA was under control of a CMV promoter. Two days after infection, 10 different fields of each culture plate were examined by comparative phase and fluorescence microscopy to determine percentage of cells expressing EGFP.

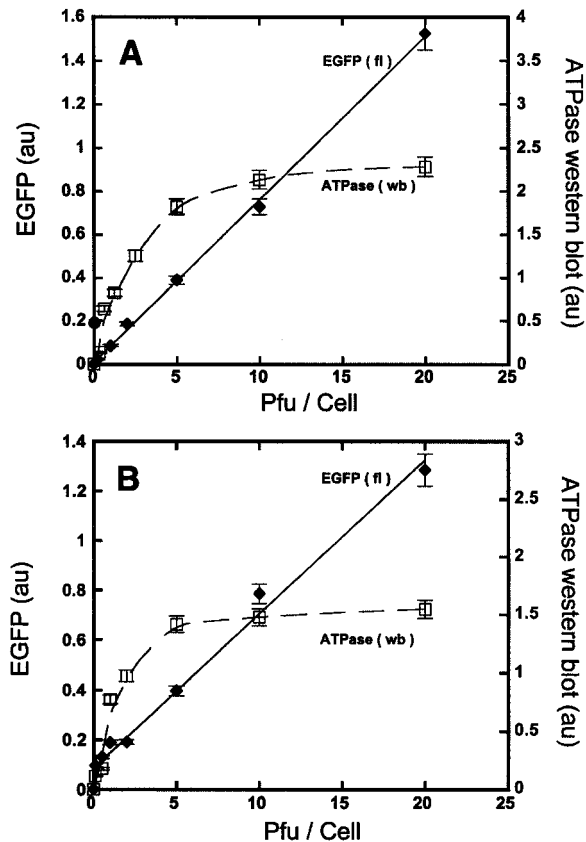


Fig. 3. Expression of EGFP (\blacklozenge) or SERCA1 (\square) in COS-1 cells (A) or chicken embryonic cardiac myocytes (B) after infection with various plaque-forming unit levels of adenovirus vectors. Cells were harvested 2 days after infection with various titers of recombinant virus and were processed for determination of expressed protein as described in MATERIALS AND METHODS. EGFP samples were assayed by fluorometry wherein cells were scraped off the plates, suspended in 50 mM NaH₂PO₄, 10 mM Tris, pH 8.0, and 200 mM NaCl, and lysed by several passes through a 22-gauge needle. Total fluorescence intensity was then measured at 488-nm excitation wavelength and 510-nm emission wavelength. SERCA-infected samples, on the other hand, were subjected to Western blot analysis. Note that in both COS-1 cells and cardiac myocytes, cell capacity to express SERCA reaches 100% of maximum at 5–10 pfu/cell ratios. However, EGFP expression as determined by fluorometry continues to increase as the viral titer is increased to 20 pfu/cell.

dence not only on the number of transfected cells but also on the number of foreign gene copies in each cell.

As opposed to EGFP, expression of SERCA ATPase was not linearly related to the plaque-forming unit level but appeared to reach an asymptote both in myocytes and in COS-1 cells as revealed by the Western blot densities plotted in Fig. 3. It is of interest that Northern blots of samples obtained under the same conditions from either COS-1 cells or cardiac myocytes show that the SERCA mRNA levels continue to increase as the plaque-forming unit titer is raised above the asymptotic limit of SERCA protein expression (compare Fig. 4 with Fig. 3). Therefore, in our experiments, the asymptotic behavior of SERCA protein expression at high plaque-forming unit levels is not due to a transcriptional limit.

Promoter efficiency and specificity. We have previously obtained expression of functional SERCA1 pro-

tein in cultured chicken embryonic myocytes infected with recombinant adenovirus vector carrying the SERCA1 DNA-coding sequence under control of either a viral (SV40) promoter or the cTnT muscle-specific promoter (15). In most of the experiments reported here we have used the highly active CMV promoter. We also performed comparative experiments with the cTnT minimal promoter (–268), a more extended segment of the cTnT promoter, including its homologous enhancer (–550) and a chimeric construct of the CMV enhancer with the cTnT minimal promoter (Fig. 5). To eliminate comparative artifacts possibly related to inaccurate plaque-forming unit levels of various recombinant adenovirus preparations, these experiments were performed by liposome-assisted transfections, using EGFP cDNA subcloned in the same plasmids but under control of various promoter constructs. The comparative levels of EGFP expressed in cardiac myocytes and fibroblasts are shown in Table 1. The greatest difference is between the CMV and the cTnT minimal promoter (–268), with the former being more active but displaying no cell specificity and the latter being weaker but totally muscle specific.

The activity of the –268 cTnT promoter can be increased eightfold by the presence of the CMV enhancer but with significant loss of cell specificity (Table 1 and Fig. 6). The CMV enhancer, by itself, yields no protein expression. It is noteworthy that the chicken cTnT promoter is just as effective in rat neonatal myocytes (Table 1) as it is in chicken embryonic myocytes. In fact, the extended –550 cTnT promoter is two to three times more active than the –268 minimal promoter in rat neonatal myocyte and yet retains total cell specificity.

It is of interest to note that despite the different efficiencies of the CMV and of the minimal (–268) cTnT promoter, the same maximal level of SERCA1 ATPase expression can be obtained in cardiac myocytes under control of either promoter. This can be accomplished by increasing the plaque-forming unit levels of adenovirus vector containing the weaker promoter to obtain the asymptotic level of protein expression by the myocytes (Fig. 7). When we compared the total SERCA protein (either isoform) level obtained under these conditions, we found them to be approximately threefold higher than the levels of endogenous SERCA2a of control myocytes. It should be pointed out that strong heterolo-

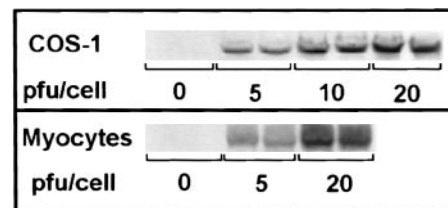


Fig. 4. Northern blots showing transcription efficiency of Ca-ATPase gene in COS-1 cells and chicken embryonic cardiac myocytes as a function of recombinant adenovirus plaque-forming unit level. For each plaque-forming unit level, duplicate experiments are shown, indicating a continuously increasing level of mRNA produced with increasing amount of adenovirus plaque-forming unit/cell ratio.

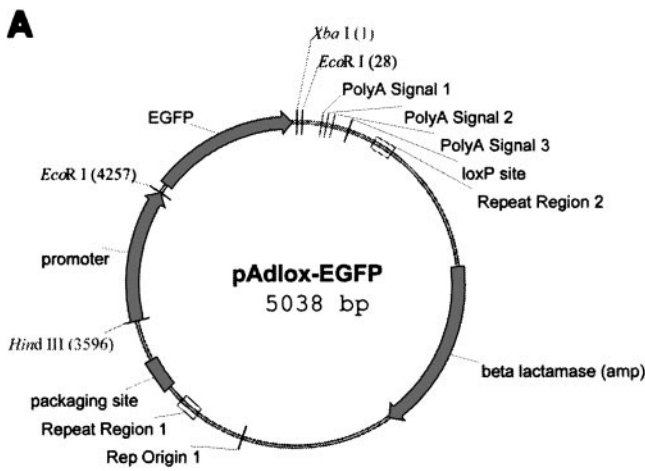


Table 1. Promoter dependence of EGFP expression

Promoter	Myocytes	Fibroblast	Species
CMV	100	100	Chicken
cTnT (-268)	7.0 ± 2.6	0	Chicken
cTnT (-550)	10.8 ± 4.1	0	Chicken
Enhancer	6.1 ± 3.1	5.2 ± 0.5	Chicken
Enh-cTnT	55.5 ± 9.8	35.1 ± 15	Chicken
CMV	100	100	Rat
cTnT (-268)	13.7 ± 5.4	0	Rat
cTnT (-550)	23.6 ± 3.1	0	Rat

Cultured chicken embryonic cardiac myocytes, rat neonatal cardiac myocytes, or skin fibroblasts were transfected with plasmids containing enhanced green fluorescence protein (EGFP) cDNA under control of various promoters as shown in Fig. 5. Transfections were performed using liposomes (chicken myocytes) or calcium phosphate (rat myocytes) as explained in MATERIALS AND METHODS. Two days after transfection, cells were harvested for determination of fluorescence. Values are given in percentage of signal developed under control of cytomegalovirus (CMV) promoter. cTnT, cardiac troponin T.

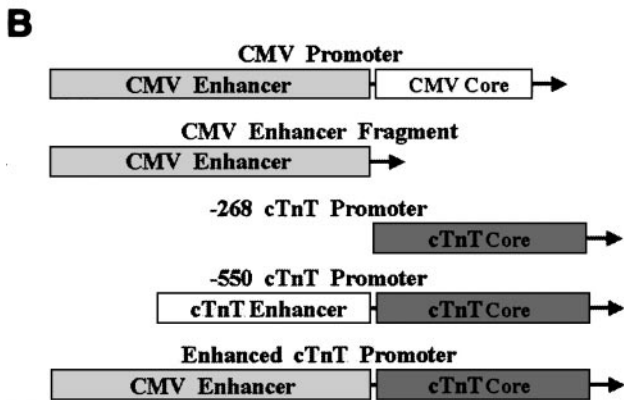


Fig. 5. A: construction of pAdlox plasmids used for transfection and/or production of recombinant adenovirus vectors. cDNA encoding EGFP or SERCA was inserted between *EcoR* I and *Xba* I sites downstream of promoter region in pAdlox. B: schematic diagram of promoter elements used in construction of pAdlox plasmid vectors. Different promoters used are 1) constitutive viral promoter of cytomegalovirus (CMV), 2) CMV enhancer, 3) muscle-specific cardiac troponin T minimal promoter region, (-268 cTnT), 4) a longer cTnT promoter segment including its homologous enhancer (-550 cTnT), and 5) CMV enhanced -268 cTnT promoter.

gous SERCA expression inhibits significantly the expression of endogenous SERCA2a (15, 22). For this reason, the high levels observed in infected cells actually display a ratio of ~7:1 for heterologous to (residual) endogenous SERCA.

SERCA1 and SERCA2a isoforms. The advantage of the CMV promoter is that high levels of expression can be obtained with relatively low adenovirus plaque-forming unit levels. In fact, we were able to obtain levels of SERCA1 and SERCA2a expression that could be demonstrated by simple staining of electrophoretic gels with Coomassie Blue (Fig. 8). Protein staining provided a very convenient method for comparative evaluation of SERCA isoform expression, independent of immunochemical reactions with antibodies that may be unequally reactive to various isoforms derived from different animal species. It should be pointed out that comparative evaluation of SERCA expression by pro-

tein staining or immunochemical detection of the same antigenic tag yielded identical results. We were then able to compare the activities of microsomes obtained from COS-1 cells infected with vectors carrying SERCA1 or the SERCA2a cDNA. The COS-1 expression system is very well suited for this comparison, because noninfected COS-1 cells sustain negligible SERCA activity. We then found that when the Ca²⁺ uptake and ATPase activity of infected cells were related to the levels of expressed enzymes (as revealed by electrophoresis and protein stain or by immunodetection of an identical antigenic tag), the SERCA1 isoforms appeared to sustain approximately twofold higher rates than the SERCA2a isoforms (Fig. 9).

The functional rates given in Fig. 9 are heavily dependent on the amount of recombinant SERCA per

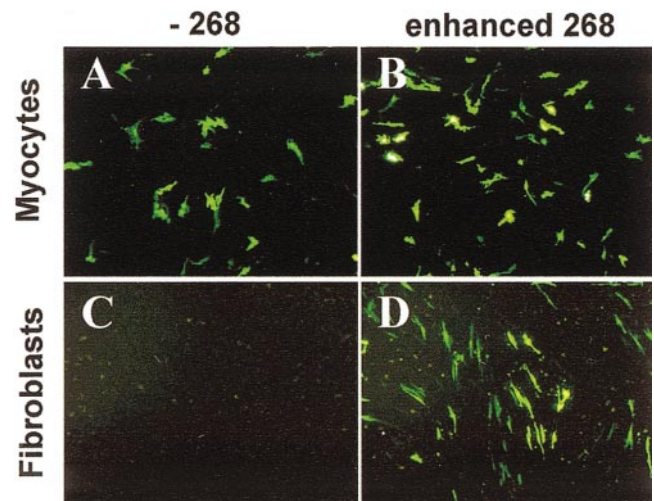


Fig. 6. Enhancement of EGFP expression in chicken embryonic cardiac myocytes by linking a CMV enhancer fragment to -268 cTnT muscle-specific promoter in transfection plasmid. Chicken embryonic cardiac myocytes (A and B) and fibroblasts (C and D) were transfected using lipid pfx8 (Invitrogen) with pAdlox-EGFP plasmids. For each type of cell, plasmids used were under control of either the -268 cTnT (A and C) or a CMV-enhanced -268 cTnT (B and D) promoter. Note that -268 cTnT promoter is 100% muscle specific because it is not expressed in fibroblasts (C). Linkage with CMV promoter causes a significant loss of specificity.

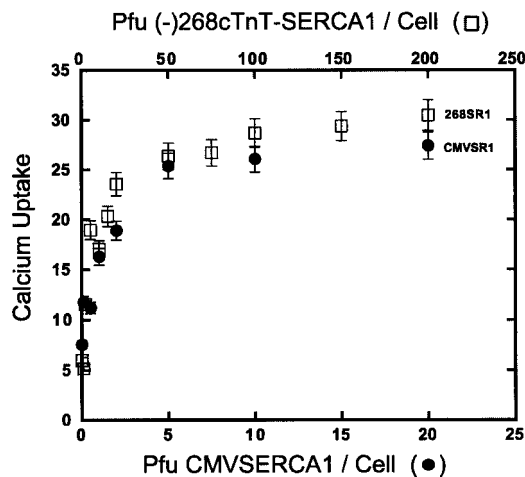


Fig. 7. Comparison of $-268cTnT$ -SERCA1 and CMV-SERCA1 expression in chicken embryonic cardiac myocytes as a function of recombinant virus plaque-forming unit level. Cells were infected with 0–20 pfu/cell of CMV-SERCA1 (lower x -axis) or 0–200 pfu/cell of $-268cTnT$ -SERCA1 (upper x -axis) recombinant adenoviruses. Two days after infection, lysates were harvested and processed for determination of calcium uptake rates as described in MATERIALS AND METHODS. Uptake was terminated at sequential times by vacuum filtration. Note that muscle-specific cTnT promoter yields functional enzyme with maximal calcium uptake rates equal to those obtained with constitutive CMV promoter. At saturation level, calcium transport rate is ~ 25 – 30 $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$, ~ 5 times the amount of endogenous SERCA (basal SERCA2 level, 5 – 8 $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$).

total microsomal protein, as revealed by electrophoresis. To eliminate the possible interference of inaccurate SERCA protein determinations, we then performed a series of experiments in which the turnover of the recombinant enzymes was determined by pulse chase. In these experiments we first allowed the enzyme to reach steady-state levels of radioactive phosphorylated enzyme intermediate by incubation with [³²P]ATP and

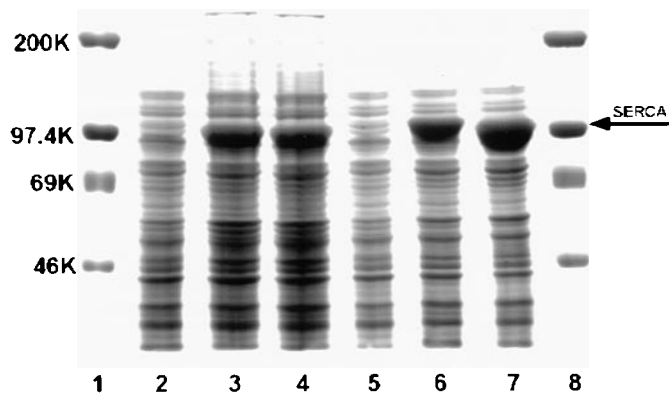


Fig. 8. SDS-PAGE of microsomal fractions of COS-1 cells infected with chicken and rabbit CMV-SERCA1 or CMV-SERCA2a recombinant adenoviruses. Cells were separately infected with 0 or 10 pfu/cell of four recombinant adenoviruses. Two days after infections, microsomal fractions were prepared as described by Strock et al. (30). Microsomal proteins were resolved in 7.5% Laemmli gels, stained with Coomassie Blue and photographed. Lanes 1 and 8: molecular mass markers. Lanes 2 and 5: uninfected cells. Lane 3: chicken SERCA1. Lane 4: chicken SERCA2a. Lane 6: rabbit SERCA1. Lane 7: rabbit SERCA2a. SERCA content in preparations shown on lanes 3, 4, 6, and 7 was determined to be 10–20% of total microsomal protein as analyzed by densitometry.

then added excess nonradioactive ATP so that hydrolytic cleavage of the radioactive intermediate would be followed by formation of nonradioactive intermediate. Under these conditions, the radioactive intermediate undergoes exponential decay with a rate constant that is representative of the enzyme turnover and is independent of the enzyme concentration. It is shown in Fig. 10 that the SERCA1 turnover is approximately two times faster than the SERCA2a turnover. In fact, the average rate constants were 0.26 and 0.14 s^{-1} for the chicken SERCA1 and SERCA2a, respectively, and 0.31 and 0.12 s^{-1} for the rabbit SERCA1 and SERCA2a. This is in contrast with previous reports (Ref. 16; see DISCUSSION).

To obtain a further comparison of SERCA1 and SERCA2a functions, we infected myocytes with increasing plaque-forming unit titers of adenovirus vectors carrying either SERCA1 or SERCA2a cDNA. We found that the asymptotic levels of function, corresponding to maximal expression, were approximately two times higher for the SERCA1 than for the SERCA2a ATPase (Fig. 11). This is again consistent with a higher turnover of the SERCA1 isoform and demonstrates that, under conditions of maximal heterologous SERCA expression, the calcium transport activity of myocytes can be increased ~ 5.0 - or 2.6 -fold, depending on whether SERCA1 or SERCA2a cDNA is used.

Ca²⁺ concentration dependence of endogenous and heterologous SERCA. Finally, we investigated whether endogenous and heterologous ATPase isoforms may be activated by different Ca²⁺ concentrations. This evaluation was obtained by measuring ATP hydrolysis by microsomal vesicles rendered leaky by the addition of a Ca²⁺ ionophore to avoid back inhibition by accumulated Ca²⁺ and allow linear rates of enzyme activity, depending on activation by Ca²⁺. It is shown in Fig. 12A that SERCA1 and SERCA2a expressed in COS-1 cells display an identical Ca²⁺ concentration dependence. On the other hand, the endogenous SERCA2a ATPase of myocytes appears to have a slightly higher Ca²⁺ dependence than heterologous SERCA1 (Fig. 12B). The difference is small but nevertheless consistent with the influence of phospholamban on SERCA activation by Ca²⁺, as demonstrated in adult cardiac muscle by several laboratories (3, 19, 27, 29, 32, 33), including our own (5). These experiments demonstrate that the different velocities displayed by various isoforms (Figs. 9, 10, and 11) are not due to a different level of activation by the same concentration of Ca²⁺ but rather to intrinsic kinetic properties of each isoform.

DISCUSSION

Regulation of several cell functions, including contraction and relaxation, is dependent on transient changes of the cytosolic Ca²⁺ concentration, as well as cytosolic Ca²⁺ sequestration by SERCA ATPases. Therefore, experimental manipulation of SERCA copy numbers is of general interest for possible effects on cell functions. To this aim, development of transgenic animal models (16, 22) is very useful inasmuch as functional consequences of SERCA manipulations can be studied at the whole organ or whole animal level (1). On the other

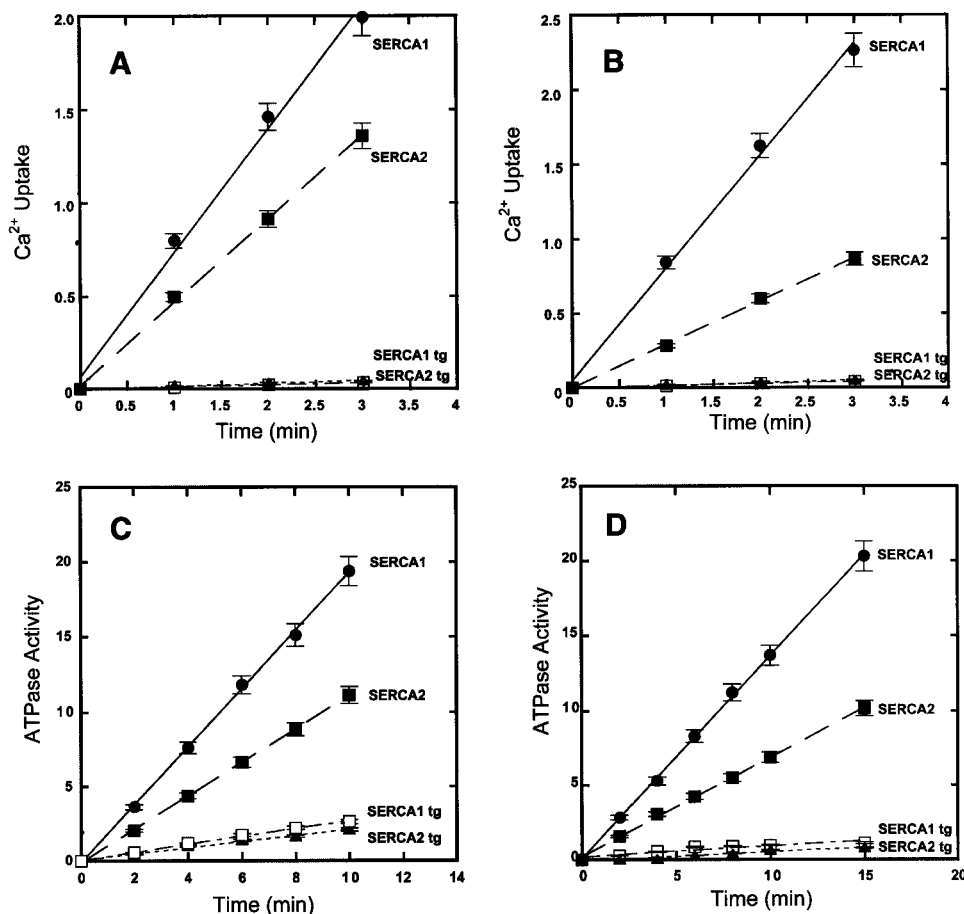


Fig. 9. Calcium uptake (A and B) and ATPase (C and D) activities of microsome fractions of COS-1 cells infected with chicken (A and C) or rabbit (B and D) SERCA1 or SERCA2a adenovirus vectors. Microsome fractions shown in Fig. 8 were assayed for calcium uptake or ATPase activity as described in MATERIALS AND METHODS. Rates of calcium uptake and ATPase activity were corrected for expression of each SERCA isoform on the basis of relative densities of electrophoretic bands stained with Coomassie Blue and by Western blotting based on a common *c-myc* tag. SERCAtg labeling refers to measurements in presence of thapsigargin, a specific SERCA inhibitor. Values are expressed in $\mu\text{mol/mg protein}$.

hand, cultured cells provide an opportunity for extensive evaluation of gene transfer techniques, promoter and cDNA constructs, gene copy numbers, and other variations that may be useful in selecting the most suitable conditions for gene transfer. We find that this type of experiment is greatly aided by recombinant adenovirus vectors. These vectors provide a most efficient and convenient method for delivery of DNA constructs to a variety of cells, including cardiac myocytes. Because the recombinant virus is replication defective, its titer can be kept constant and adjusted to introduce a predetermined number of heterologous gene copies in cultured cells, thereby producing controlled expression of recombinant protein. In fact, we demonstrated with our experiments that with an average of 10 pfu per seeded cell, it is possible to infect nearly all COS-1 cells or myocytes in culture (Fig. 2). Further increase in the multiplicity of infection produces a proportionally higher level of expression, evidently due to the higher number of gene copies introduced in each cell.

We find that while the expression level of cytosolic EGFP rises linearly with the adenovirus vector titer up to 20 pfu per seeded cell, the expression level of membrane bound SERCA reaches an asymptote at 10 pfu per seeded cell (Fig. 3). This is true in both COS-1 cells and myocytes, although the maximal SERCA expression in COS-1 cells is somewhat higher than that in myocytes. It is of interest that SERCA mRNA

continues to increase as the viral titer is raised (Fig. 4), indicating that the promoters used in our experiments can overcome the limits of endogenous transcription regulation. It is then apparent that posttranscriptional events impose a limit on the level of SERCA expression. These observations explain previous studies on Ca²⁺-ATPase overexpression in transgenic animals, in which the SERCA2a protein level was found to increase only 1.3- to 1.5-fold, whereas the mRNA level was increased 3.9- to 7.9-fold (1). In our experiments with cardiac myocytes infected with adenovirus vectors, the level of SERCA protein could be raised to a threefold maximum compared with the level of endogenous SERCA in control myocytes. It should be pointed out the heterologous SERCA is sevenfold higher compared with the (residual) endogenous SERCA of the same infected myocytes because of reduction of endogenous SERCA following infection. This rise is higher than obtained in any of the transgenic animal models and defines the maximal level that can be reached by manipulation of SERCA expression.

The choice of a promoter is a cogent question, because one would want a strong as well as cell-specific promoter, to obtain high rates of transcription and yet avoid expression in cells other than myocytes. We found the CMV promoter to be very strong but not at all cell specific. As previously reported (15), the minimal segment of the cTnT promoter (24) can be used for heterologous expression of SERCA ATPase. Its short

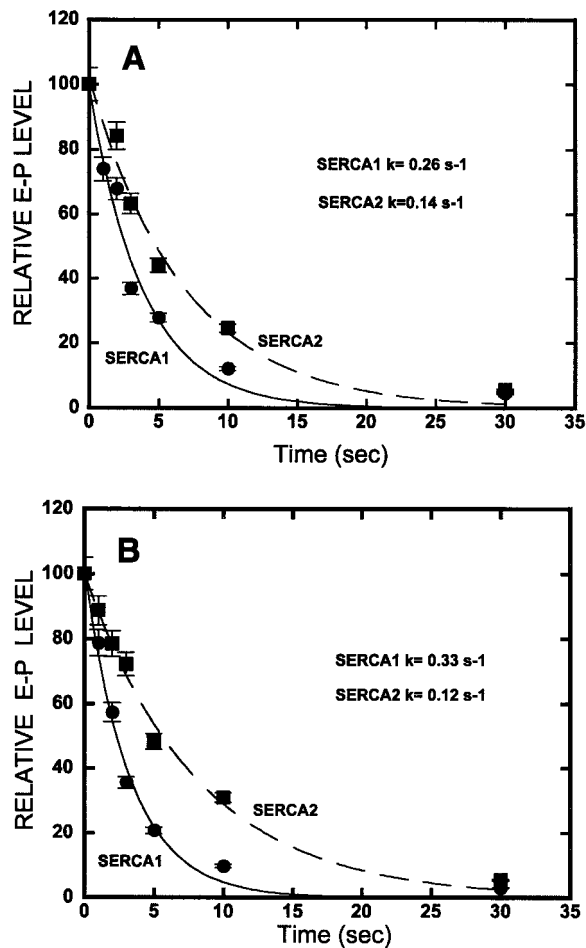


Fig. 10. Decay of phosphorylated intermediates formed by microsomal fractions of COS-1 cells infected with chicken SERCA1 and SERCA2a (A) or rabbit SERCA1 and SERCA2a (B) adenovirus vectors. Microsomal fractions shown in Fig. 8 were incubated with [³²P]ATP to form steady-state levels of phosphorylated enzyme intermediate. After 10 s, a pulse chase of nonradioactive ATP excess was added, and serial samples were then quenched with perchloric acid. Amount of residual radioactive intermediate remaining was determined by autoradiography and phosphoimaging of electrophoretic gels. Decay rate constants were calculated by fitting data with a single-exponential equation. It should be noted that calculated decay rates are independent of enzyme concentration.

length (268 bp) and its stringent myocyte specificity are very convenient, although its efficiency is ~10 times lower than that of the CMV promoter (Table 1). At any rate, an important conclusion of our experiments is that it is possible to reach maximal levels of SERCA expression using either the CMV or the cTnT promoter (Fig. 7), if the titer of the adenovirus vector is adjusted to deliver a suitable number of gene copies. As noted above, no further SERCA expression can be obtained when the asymptotic levels of translation and targeting are reached, even in the presence of higher transcriptional pressure.

It should be noted that increasing the length of the -268 bp cTnT promoter to -550 bp, thereby including homologous enhancing segments (24), raised significantly the strength of the minimal promoter, especially in neonatal rat myocytes. On the other hand, inclusion

of the CMV enhancer greatly increased the strength of the -268 cTnT promoter but also caused considerable loss of cell specificity (Table 1 and Fig. 6). This observation is of interest inasmuch as it shows that a nonspecific viral promoter enhancer, which has no activity of its own, can form a very active transcriptional complex in concomitance with the minimal cardiac promoter, evidently interacting with constitutive cardiac factors. The other observation of interest is that the chicken cTnT promoter is active in rat as well as in chicken myocytes.

Comparative kinetic definition of the skeletal and cardiac SERCA isoforms has been a problem since early studies (14) showed that sarcoplasmic reticulum (SR) vesicles isolated from cardiac muscle were less active than SR vesicles isolated from skeletal muscle. Even though better SR preparations were later obtained, we were left with the impression that isolation of SR vesicles from cardiac muscle produces more protein degradation and more contaminants than isolation from skeletal muscle and, for this reason, the cardiac vesicles seem less active. However, the more recently

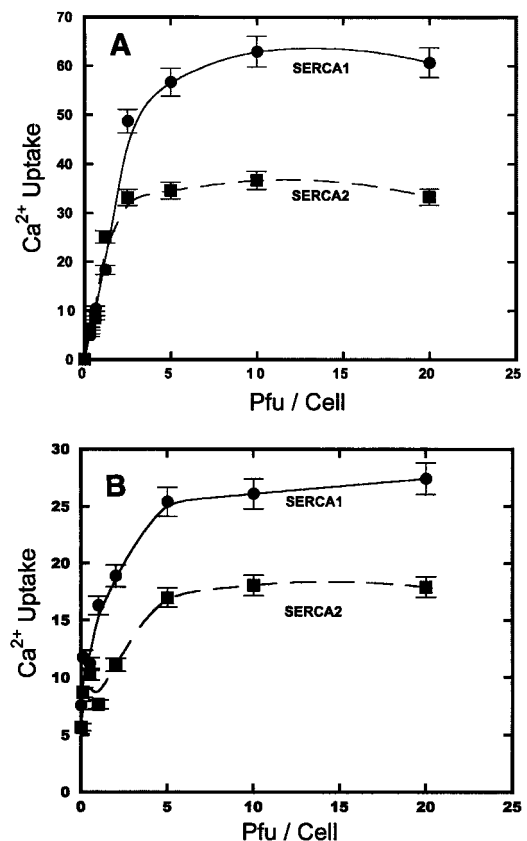


Fig. 11. ATP-dependent calcium uptake of cell lysates of COS-1 cells (A) or chicken embryonic cardiac myocytes (B) infected with increasing plaque-forming unit levels of SERCA1 and SERCA2a adenovirus vectors. Cells were infected with increasing plaque-forming unit levels of SERCA1 and SERCA2a adenovirus vectors, and cell lysates were processed for calcium uptake activity as described in MATERIALS AND METHODS. Note that 1) maximal uptake of myocytes is lower than that of COS-1 cells, 2) SERCA2a uptake is always lower than SERCA1 uptake, and 3) control cardiac myocytes (0 pfu) have a baseline calcium uptake due to endogenous SERCA2a. Values are expressed in $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$.

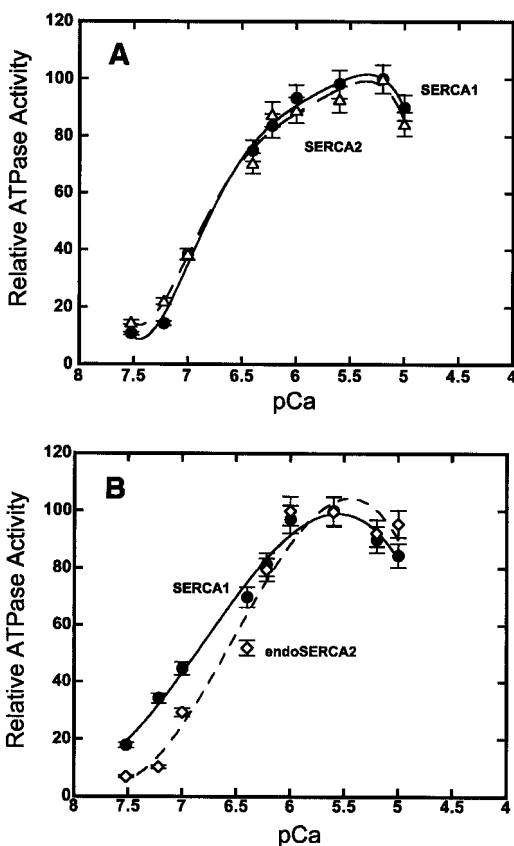


Fig. 12. Calcium concentration dependence of SERCA ATPase activity. *A*: heterologous SERCA1 and SERCA2a expressed in COS-1 cells. *B*: endogenous SERCA2a and heterologous SERCA1 expressed in chicken embryonic cardiac myocytes. Cells were infected with either 0 or 10 pfu/cell of appropriate adenovirus vector, and cell lysates were processed for functional analysis as described in MATERIALS AND METHODS. ATPase activity as a function of free Ca²⁺ concentration was measured in each sample. Free Ca²⁺ was obtained with a Ca-EGTA buffer and calculated according to Fabiato and Fabiato (6). Note that heterologous SERCA1 and SERCA2a display same Ca²⁺ concentration dependence. Ca²⁺ activation curve for endogenous SERCA2a appears slightly shifted to right.

acquired possibility of expressing SERCA protein by transfection of COS-1 cells (25) allows us to obtain the two isoforms from the same source, thereby permitting a more rigorous comparison of their kinetic features. Our experiments demonstrate that SERCA2a turnover is approximately one-half as fast as that of the SERCA1 enzyme. This was demonstrated by comparing the steady-state velocity of preparations of which SERCA1 and SERCA2a contents were determined by the use of a common immunogenic tag or by protein stain of electrophoretic gels (Figs. 8 and 9). The turnover of the two isoforms was also evaluated by comparing the exponential (thereby concentration independent) decay of the phosphorylated enzyme intermediate (Fig. 10) and found to be twice as fast for the SERCA1 isoform. This is in contrast with the identical phosphoenzyme decay reported for endogenous SERCA2a and heterologous SERCA1 in transgenic animals (16). It should be noted, however, that these transgenic animals exhibited only a 1.8-fold increase in Ca²⁺ uptake activity, relative to controls, and the kinetic measurements included a high

component related to endogenous SERCA2a. On the other hand, we measured phosphoenzyme decay of SERCA1 and SERCA2a isoforms expressed separately by COS-1 cells, which do not have significant levels of endogenous SERCA activity. Furthermore, we repeated these measurements with both chicken and rabbit SERCA isoforms to avoid ambiguities that may be related to species differences.

Experiments with cultured myocytes (9, 10) as well as with transgenic animals (7, 13, 16, 22) have already demonstrated that heterologous expression of the SERCA1 skeletal muscle isoform can be obtained in cardiac muscle, thereby contributing to sequestration of Ca²⁺ involved in excitation-contraction coupling. In this regard we find that if expression of either isoform is obtained at maximal level by suitable adjustment of the adenovirus vector titer, the resulting Ca²⁺ transport activity is approximately two times higher in myocytes transfected with SERCA1 cDNA than in myocytes transfected with SERCA2a cDNA (Fig. 11). Therefore, under conditions permitting maximal (and nearly identical) levels of either SERCA1 or SERCA2a heterologous expression, the resulting increase in calcium transport activity is 5.0- or 2.6-fold, depending on whether the expressed isoform is SERCA1 or SERCA2a. This defines the maximal increment of Ca²⁺ transport activity that can be obtained in myocytes by expression of heterologous SERCA.

Finally, we find that the SERCA1 and SERCA2a isoforms expressed in COS-1 cells display an identical Ca²⁺ concentration dependence. COS-1 cells do not contain significant levels of endogenous SERCA or phospholamban; therefore, they are well suited to comparative experiments on the Ca²⁺ dependence of the two isoforms. On the other hand, cardiac myocytes contain significant levels of endogenous SERCA2a, which requires a slightly higher Ca²⁺ for activation (Fig. 12*B*), likely due to regulation by phospholamban. The important point is that, at least in our experiments with embryonic myocytes, neither heterologous SERCA1 nor SERCA2a appears to acquire regulation by phospholamban, because it would be revealed by a displacement of the Ca²⁺ activation curve. It should be pointed out that Ji et al. (16) found evidence of SERCA1 regulation in the heart of adult transgenic animals. They also found, however, that phospholamban expression was not increased in the same transgenic hearts (22). It is possible that the native SR content of endogenous phospholamban was sufficient to interact with the rather modest level of SERCA1 expression in those transgenic animals. In our experiments we also found no increase in either phospholamban or calsequestrin expression (results not shown).

In conclusion, we obtained basic information on effective methods for SERCA gene transfer in cultured COS-1 cells and cardiac myocytes, choice of promoters for efficient and cell-specific transcription, limits of maximal expression, functional competence of the expressed protein, and comparative evaluation of cardiac and skeletal isoforms. This information is a basic

prerequisite for modification of cell function by gene transfer in experimental studies or possible therapeutic interventions. Further studies are required to apply the information obtained with embryonic and neonatal myocytes to adult cardiac muscle.

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