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Ca^{2+}-ATPases in non-failing and failing heart: evidence for a novel cardiac sarco/endoplasmic reticulum Ca^{2+}-ATPase 2 isoform (SERCA2c)

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We recently documented the expression of a novel human mRNA variant encoding a yet uncharacterized SERCA [SR (sarcoplasmic reticulum)/ER (endoplasmic reticulum) Ca^{2+}-ATPase] protein, SERCA2c [Gélébart, Martin, Enouf and Papp (2003) Biochem. Biophys. Res. Commun. **303**, 676–684]. In the present study, we have analysed the expression and functional characteristics of SERCA2c relative to SERCA2a and SERCA2b isoforms upon their stable heterologous expression in HEK-293 cells (human embryonic kidney 293 cells). All SERCA2 proteins induced an increased Ca^{2+} content in the ER of intact transfected cells. In microsomes prepared from transfected cells, SERCA2c showed a lower apparent affinity for cytosolic Ca^{2+} than SERCA2a and a catalytic turnover rate similar to SERCA2b. We further demonstrated the expression of the endogenous SERCA2c protein in protein lysates isolated from heart left ventricles using a newly generated SERCA2c-specific antibody. Relative to the known uniform distribution of SERCA2a and SERCA2b in cardiomyocytes of the left ventricle tissue, SERCA2c was only detected in a confined area of cardiomyocytes, in close proximity to the sarcolemma. This finding led us to explore the expression of the presently known cardiac Ca^{2+}-ATPase isoforms in heart failure. Comparative expression of SERCAs and PMCAs (plasma-membrane Ca^{2+}-ATPases) was performed in four non-failing hearts and five failing hearts displaying mixed cardiomyopathy and idiopathic dilated cardiomyopathies. Relative to normal subjects, cardiomyopathic patients express more PMCAs than SERCA2 proteins. Interestingly, SERCA2c expression was significantly increased (166 ± 26 %) in one patient. Taken together, these results demonstrate the expression of the novel SERCA2c isoform in the heart and may point to a still unrecognized role of PMCAs in cardiomyopathies.

Key words: endoplasmic reticulum, heart failure, human embryonic kidney 293 cell (HEK-293 cell), isoform, plasma membrane Ca^{2+}-ATPase (PMCA), sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA).

INTRODUCTION

Cell Ca^{2+} signalling is a dynamic oscillatory process regulating a variety of important cellular functions such as secretion, contraction and gene transcription. The free Ca^{2+} concentration is strictly controlled in space, time and amplitude [1]. Ca^{2+} homoeostasis is dependent on the activity of several molecular players, which mediate uptake and release of both extracellular and intracellular Ca^{2+}.

Specialized Ca^{2+} pumps such as the SPCAs (secretory-pathway Ca^{2+}-ATPases), the SERCAs [SR (sarcoplasmic reticulum)/ER (endoplasmic reticulum) Ca^{2+}-ATPases] and the PMCAs (plasma-membrane Ca^{2+}-ATPases) can take up intracellular Ca^{2+} [2–7].

The human genes *ATP2A1-3*, *ATP2B1-4* and *ATP2C1-2* encode the SERCA, PMCA and SPCA enzymes respectively [2–7]. Each gene gives rise to alternatively spliced isoforms. Alternative splicing of the pre-mRNA from all PMCA genes may generate up to 30 variants of theoretically possible isoforms. PMCA1 and PMCA4 gene products are expressed in virtually all organs, tissues and cell types. PMCA1b and PMCA4b are known as the 'housekeeping' isoforms. Until recently, *SERCA1* and *SERCA2* genes were known to generate two isoforms per gene, which differ in their C-termini. They are mainly expressed in adult (SERCA1a) and neonatal (SERCA1b) skeletal muscles, in cardiac muscle (SERCA2a) and in all cell types (SERCA2b). Quite recently, a new SERCA2c mRNA was described [6]. The third gene, *SERCA3*, was also recently shown to have various 3′-end splice variants encoding species-specific isoforms with different C-termini: SERCA3b–SERCA3f in human, SERCA3b–SERCA3c in mouse, and SERCA3b–SERCA3c in rat proteins [7–11], in addition to the species-non-specific SERCA3a isoform [8,12,13]. The SERCA3 isoforms are differently co-expressed in a variety of cells and tissues including muscle and non-muscle tissues [7–13].

Such an increasing plurality of Ca^{2+}-ATPase isoforms may provide additional possibilities for cells to tightly regulate their Ca^{2+} signals. Indeed, although the rationale for such a diversity of Ca^{2+}-ATPase isoforms is still not understood, overview of known data is found in recent reviews [2,4]. For PMCAs, functional differences include the apparent affinities towards Ca^{2+} and calmodulin or regulation by lipids and phosphorylation [4]. Steady-state and transient kinetic analyses have been recently

Abbreviations used: ER, endoplasmic reticulum; $[Ca^{2+}]_C$, cytosolic Ca^{2+} concentration; $[Ca^{2+}]_{ER}$, ER Ca^{2+} content; fura 2/AM, fura 2 acetoxymethyl ester; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK-293 cell, human embryonic kidney 293 cell; nNOS, neuronal nitric oxide synthase; PMCA, plasma-membrane Ca^{2+}-ATPase; RT, reverse transcriptase; SR, sarcoplasmic reticulum; SERCA, SR/ER Ca^{2+}-ATPase; SPCA, secretory-pathway Ca^{2+}-ATPase.

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performed to functionally characterize and explain the overall biochemical differences (e.g. the affinity for cytosolic Ca^{2+} and the turnover rate) between SERCA1, SERCA2 and SERCA3 isoforms [14,15] on one hand, and, on the other hand, between SERCA1 and SPCA1 pumps [16]. Taken together, the distinct locations (plasma membrane, SR, ER and Golgi apparatus), the differential tissue and cell distribution and the differently regulated expression of the Ca^{2+} pump isoforms lend physiological significance for the maintenance of this protein diversity. The recent identification of various partners interacting with PMCAs has given insights into the putative functions of Ca^{2+} pumps as molecular organizers and regulators of Ca^{2+}-dependent signal transduction pathways. Molecular genetic studies of defective genes in humans, coupled with the observation that distinct phenotypes were found in knockout models, point to compensatory phenomena [3,17].

Since SERCA isoform diversity appears to be extremely important for the cellular and organism functions, we have now investigated in depth the expression (at both mRNA and protein level) and the functional characteristics of the newly identified SERCA2c variant relative to SERCA2a and SERCA2b. The corresponding cDNA clones were constructed and the recombinant SERCA2 proteins from stably transfected HEK-293 cells (human embryonic kidney 293 cells) have been studied for their Ca^{2+} pumping activity in both intact cells and in isolated vesicles mainly consisting of internal ER and Golgi membranes (microsomes). Expression of SERCA2c mRNA was further screened in different tissues. In addition, a SERCA2c-specific antibody has been generated. At the mRNA level, SERCA2c appears to be mainly expressed in heart tissue. Relative to SERCA2a and SERCA2b, SERCA2c protein presents a distinct localization in left ventricle of normal hearts. Finally, because heart failure is often associated with abnormal Ca^{2+} handling and since contradictory results have been reported [18,19] concerning the regulation of SERCA2a, we have also monitored the expression of the SERCA2 and (still unexplored) PMCA isoforms in some patients with a failing heart.

EXPERIMENTAL

Patients

With the permission of the next-of-kin we collected left-ventricular myocardium from seven brain-dead organ donors who had died from head trauma or cerebrovascular accidents and from seven patients with diverse cardiomyopathies. Mean age of the non-failing group was 58 ± 12 years and all were selected on the basis of clinical history or any instrumental data available of no cardiac disease. Mean age of patients with failing hearts was 31 ± 16 years and all had NYHA (New York Heart Association) class III or IV congestive heart failure. Samples were immediately frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ until use. Our studies comply with the Declaration of Helsinki and institutional ethical regulations.

Cell culture

HEK-293 cells were obtained from the A.T.C.C. (Manassas, VA, U.S.A.) and cultured in RPMI 1640 medium with Glutamax-1 supplemented with 10 % (v/v) heat-inactivated foetal calf serum.

Isolation of total RNA and protein from human myocardium and HEK-293 cells

Myocardium from the left ventricular wall was powdered in liquid nitrogen. Total RNA and protein were prepared using TRIzol® reagent (Gibco BRL) and the procedure recommended by the manufacturer. Aliquots were stored at $-20\,^{\circ}C$ until use.

Preparation of recombinant HEK-293 cell membrane proteins

Isolation of membrane fractions enriched in intracellular membranes (100000 ***g*** fractions) from HEK-293 cells was performed as described in [9,11].

Antibodies

All human SERCA2 proteins were visualized using the monoclonal antibody IID8 [9], or specific polyclonal antibodies against SERCA2a and SERCA2b [20] and SERCA2c (see below). The anti-calreticulin polyclonal antibody (BioMol, Plymouth Meeting, PA, U.S.A.) and the anti-α-actinin monoclonal antibody (Sigma, St. Louis, MO, U.S.A.) were also used. The human PMCAs and PMCA4b isoform were visualized with the 5F10 [9] and the JA3 respectively [21] (Neomarkers, Fremont, CA, U.S.A.) monoclonal antibodies. Secondary anti-rabbit and anti-mouse horseradish peroxidase-conjugated antibodies for immunoblottings were obtained from Jackson Immunoresearch (West Grove, PA, U.S.A.). Secondary anti-rabbit FITC fluorochrome antibody, biotinylated anti-rabbit antibody and streptavidin–FITC as well as anti-mouse antibody conjugated with Texas Red for immunochemistry were from Amersham Biosciences (Little Chalfont, Bucks., U.K.).

Generation and characterization of a novel human SERCA2c-specific polyclonal antibody

A SERCA2c-specific polyclonal antibody was generated by immunizing SPF rabbits with a mixture of the P1 and P2 peptides indicated in Figure 1(B) (Eurogentec, Herstal, Belgium). Eurogentec also performed the affinity purification of antiserum using each peptide. Purified anti-SERCA2c-P1 and anti-SERCA2c-P2 antibodies were tested by immunoblotting using recombinant SERCA2c protein (results not shown). The anti-SERCA2c-P1 antibody presented the highest immunoreactivity and was used for all immunoblotting and immunofluorescence experiments described in the present study.

Western-blot analyses

Protein electrophoresis and Western-blots analyses were performed as described in [9] for the IID8 and 5F10 antibodies, except that proteins were loaded on to Novex Tris/glycine 8 % gels (Invitrogen). After electrophoresis, the separated proteins were transferred on to nitrocellulose membranes. For SERCA2 proteins, nitrocellulose membranes were incubated with a 1:1000 dilution of each of the antibodies against SERCA2a, SERCA2b and SERCA2c in Tris-buffered saline (10 mM Tris/HCl and 150 mM NaCl, pH 7.4), 5 % (w/v) non-fat milk and 0.1 % Tween 20 for 2 h at room temperature (25 °C). For PMCA4b protein and calreticulin, nitrocellulose membranes were incubated with a 1:200 dilution of JA3 and a 1:1000 dilution of anti-calreticulin antibodies respectively in the buffer described above. After washing, blots were treated with a 1:10000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (SERCA2a–SERCA2c and calreticulin), or a 1:2000 (IID8 and 5F10) or a 1:4000 (JA3) dilution of horseradish peroxidase-conjugated anti-mouse IgG, for 1 h 30 min. Antibody binding was revealed using enhanced chemiluminescence Western blotting reagents according to the manufacturer's instructions (Amersham Biosciences). Luminograms were scanned using Adobe Photoshop 7.0 and, where indicated, quantified by Molecular Analyst, version NIH Image 1.62b7.

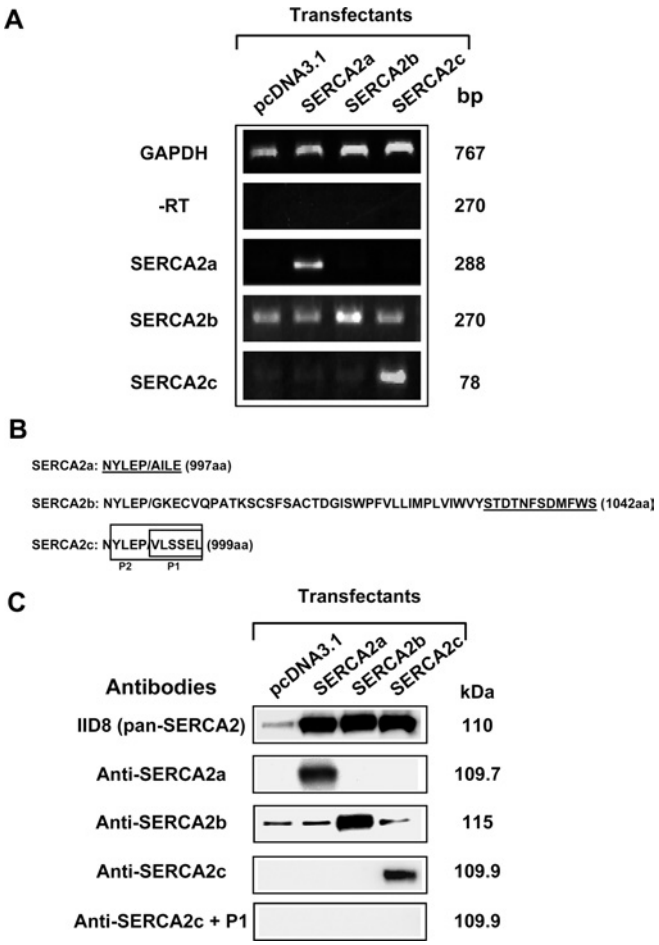


Figure 1 HEK-293 cells stably transfected with SERCA2a, SERCA2b and SERCA2c cDNA constructs overexpress the corresponding recombinant proteins

(**A**) Comparison of the stably transfected SERCA2 proteins at mRNA level. RT reactions ($n = 5$) were carried out starting from 250 ng of template mRNA. Subsequent PCR reactions ($n = 5$) were performed for GAPDH and SERCA2b for 19 cycles, and for SERCA2a and SERCA2c for 20 cycles. No DNA contamination was observed when SERCA2b mRNA amplification was performed in the absence of reverse-transcribed template (-RT). The numbers indicate the sizes of PCR products in base pairs. (**B**) C-termini of SERCA2 proteins showing the epitopes (underlined or boxed sequences) for SERCA2-specific polyclonal antibodies [6,20]. 'Solidus' marks the splice sites. The boxed amino acid sequences represent the two peptides (P1 and P2) used for SERCA2c-specific immunization. (**C**) Comparison of the stably transfected SERCA2 proteins at protein level. Membrane proteins were isolated from HEK-293 cells transfected with empty vector pcDNA3.1, SERCA2a, SERCA2b and SERCA2c cDNAs. Microsomes (10 μg) were used for Western blotting using the indicated antibodies. To test the specificity of the anti-SERCA2c antibody, the blots were treated either in the absence or presence of 10 μM peptide (P1) ($n = 3$). The numbers indicate protein molecular masses (in kDa).

Immunofluorescent histochemistry

Samples of two human left ventricular myocardium were frozen in isopentane at -155 °C. Serial ventricular cryosections (7 μm) were fixed and permeabilized with acetone/methanol (1:1, for 15 min at -20 °C), saturated with 5 % (w/v) BSA in PBS (30 min at room temperature), incubated with monoclonal anti-α-actinin antibody (1:50 in 2 % BSA–PBS for 30 min at 37 °C), washed in 1 × PBS, and incubated with primary antibodies anti-SERCA2a, anti-SERCA2b or anti-SERCA2c (1:50, 1:10 and 1:10 respectively, in 2 % BSA–PBS for 30 min at 37 °C). After three rinses in PBS, the sections were successively incubated with secondary antibodies for 20 min at room temperature. For the anti-α-actinin antibody, we used the anti-mouse antibody conjugated with Texas Red (1:50 dilution in PBS containing 1:25 serum of rat). Then, for the anti-SERCA2a antibody, we used the anti-rabbit FITC fluorochrome antibody (1:40 dilution in PBS containing 1:25 serum of rat). For anti-SERCA2b and anti-SERCA2c antibodies, we used the biotinylated anti-rabbit antibody (1:50 dilution in PBS containing 1:25 serum of rat) followed by streptavidin–FITC (1:40 in PBS). Secondary antibodies alone did not reveal any significant fluorescence signal after the incubation with the tissue slices. Fluorescence was visualized using a Leica DMR (Digital Modul-R) microscope equipped with a JVC (Victor Company of Japan) colour video camera KY-F50.

RT (reverse transcriptase)–PCR

For total RNA extraction and RT–PCR experiments, essentially identical methods as in [9,11] were used. The primers (Genosys Sigma–Aldrich, St. Louis, MO, U.S.A.) listed in Supplementary Table 1 (at http://www.BiochemJ.org/bj/395/bj3950249add.htm) have been used to amplify GAPDH (glyceraldehyde-3-phosphate dehydrogenase), all SERCA2, PMCA1c–PMCA1b and PMCA4a–PMCA4b mRNAs. PCR was performed as described in [9,11,22] except for the touch down-PCR experiment, which was performed for 10 cycles with annealing temperature decrement from 65 to 55 °C (for multiple tissue cDNAs from human cardiovascular system) or from 59 to 55 °C (for multiple tissue cDNAs). GAPDH amplifications were used as internal RNA controls. PCR products were visualized and quantified as previously described [9,11].

Plasmid construction

For expression constructs, the cDNA encoding the SERCA2b in pcDNA3.1 was used [9]. 3′-End variant-specific SERCA2a and SERCA2c cDNAs were generated by RT–PCR amplification using RNAs isolated from human heart and U937 cells respectively. For this, a common forward primer: 5′-GCATGCTGGATCCTCCGA-3′ and two reverse primers: 5′-GGAAGCGGTTACTCCAG-3′ and 5′-TCTAGAGCAGCAGAGCAGGAGCCTT-3′ were used for SERCA2a and SERCA2c respectively. Specific products were then XhoI/XhoI (SERCA2a) or BamHI/NotI (SERCA2c) excised after oriented subcloning in pCR2.1 vector. SERCA2a and SERCA2c cDNAs were created by switching the XhoI/XhoI (SERCA2a) or BamHI/NotI (SERCA2c) fragments of the full-length pcDNA3.1/SERCA2b construct with the 3′-end variant-specific products. The sequences of the constructs were verified by automated dye terminator sequencing (Applied Biosystems AB1 100 model 377; Genome Express, Grenoble, France).

Stable transfections

cDNAs for transfections were purified using a plasmid purification kit (Macherey-Nagel, Düren, Germany). Cells were transfected with 10 μg of the cDNAs using the transfection agent ExGen 500 (Euromedex, Souffelweyersheim, France) accordingly [9,11]. Transfected HEK-293 cells were treated for isolation of total RNA and protein or for fractions enriched in intracellular membranes (microsomes).

Measurements of cytosolic Ca^{2+} concentration $[Ca^{2+}]_C$ and $[Ca^{2+}]_{ER}$ (ER Ca^{2+} content)

Confluent HEK-293 cells (2×10^6 cells) were loaded with 1.75 μM fura 2/AM (fura 2 acetoxymethyl ester) and studied for $[Ca^{2+}]_C$ and $[Ca^{2+}]_{ER}$ as described previously [9,11]. Fluorescence measurements were performed using a Shimadzu RF-1501 spectrofluorimeter (Shimadzu Europe, Diusberg, Germany). Ratios (R)

at 510 nm fluorescence emission obtained at 340 and 380 nm excitation wavelengths were calculated. Calibrations were performed by addition of $CaCl_2$ or EGTA to obtain R_{max} and R_{min} values respectively. Levels of $[Ca^{2+}]$ were calculated from the binding equation: $[Ca^{2+}] = K_d\beta\ (R - R_{min})/(R_{max} - R)$.

Study of catalytic cycle

The turnover rate for ATP hydrolysis and the phosphorylation from [γ-^{32}P]ATP were determined as described in [14–16]. The rate of ATP hydrolysis was measured [14] at 37 °C and pH 7.0 under conditions where Ca^{2+} activation was maximal. The molecular turnover rate was calculated as the ratio of the Ca^{2+}-activated ATPase activity to the amount of active enzyme measured as the capacity for phosphorylation [14]. Phosphorylation from [γ-^{32}P]ATP was carried out for 15 s at 0 °C in a medium containing 40 mM Mops/Tris (pH 7.0), 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 5 μM [γ-^{32}P]ATP and various concentrations of $CaCl_2$ to set the free Ca^{2+} concentration indicated. The phosphoenzyme was quenched with 0.5 vol. of 25 % (w/v) trichloroacetic acid containing 100 mM H_3PO_4. The acid-precipitated protein was washed by centrifugation and subjected to SDS/PAGE in a 7 % polyacrylamide gel at pH 6.0, and the radioactivity associated with the separated Ca^{2+}-ATPase band was quantified by imaging, using a Packard Cyclone™ Storage Phosphor system [14–16, 23,24]. Appropriate background phosphorylation levels were subtracted, and data analysis was performed as described earlier [15].

Data analysis

All results represent the means for at least three separately performed experiments. Results are shown as means ± S.E.M. Statistical significance was determined in multiple comparisons among independent groups of data in which ANOVA and the F test indicated the presence of significant differences. A value of $P < 0.05$ was considered significant.

RESULTS

Stable expression of recombinant human SERCA2c isoform

We recently described a novel human SERCA2c mRNA [6]. However, evidence for the SERCA2c protein was lacking. To study the new isoform and compare it with human SERCA2a and SERCA2b [9], SERCA2a and SERCA2c cDNAs were constructed and stably expressed in HEK-293 cells. Recombinants were tested for protein expressions by SDS/PAGE followed by Western blotting using the pan-SERCA2 antibody IID8. About seven to eight positive clones showed high expression levels compared with HEK-293 cells transfected with empty vector pcDNA3.1 (results not shown). Figure 1 characterizes one of the HEK-293 cell clones at mRNA (panel A) and protein (panel C) levels in comparison with HEK-293 cells transfected with control empty vector (pcDNA3.1). RNA studies (Figure 1A) showed the significant and specific increases in all SERCA2 mRNAs as expected. The SERCA2 recombinants were also studied for protein expressions by SDS/PAGE followed by Western-blot analysis. For Western blotting, the same pan-SERCA2 antibody, IID8, was used to estimate transfection efficiencies. SERCA2 isoform-specific antibodies were used as further controls. Figure 1(B) shows the C-termini of the SERCA2 isoforms and the epitopes of the isoform-specific antibodies. As judged by the use of the monoclonal antibody IID8, the levels of recombinant SERCA2 proteins (Figure 1C) showed similar transfection efficiencies compared with that of the empty vector pcDNA3.1 (7–8-fold increase).

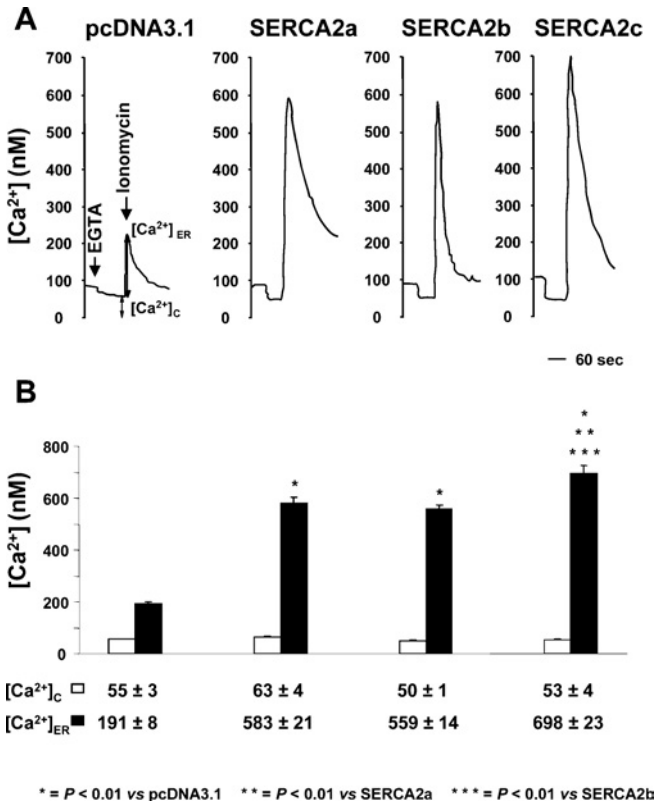


Figure 2 Recombinant SERCA2a, SERCA2b and SERCA2c proteins modulate $[Ca^{2+}]_{ER}$

Measurements of Ca^{2+} levels have been performed in the cytosol of HEK-293 cells transfected with empty vector pcDNA3.1, SERCA2a, SERCA2b and SERCA2c cDNAs as described in [9,11]. (**A**) $[Ca^{2+}]_C$ was recorded in the absence and presence of 2 mM EGTA, and the Ca^{2+} response evoked by 5 μM ionomycin was taken as an estimate of $[Ca^{2+}]_{ER}$. Arrows show the $[Ca^{2+}]_C$ and $[Ca^{2+}]_{ER}$ measurements. The double-headed arrow shows the $[Ca^{2+}]$ differences used for calculations. (**B**) Quantitative comparison of the $[Ca^{2+}]_C$ (white bars) and $[Ca^{2+}]_{ER}$ (black bars) of the SERCA2 recombinants (means ± S.E.M. for $n = 9$). *P, **P and ***$P < 0.01$ compared with pcDNA3.1, SERCA2a and SERCA2b transfectants respectively.

Each isoform-specific antibody recognized only its corresponding SERCA2 isoform. For the novel anti-SERCA2c antibody, the signal was abolished in the presence of 10 μM of the synthetic immunogenic P1 peptide. The electrophoretic migrations of the SERCA2 recombinant proteins were in agreement with their predicted molecular masses, thus confirming the intactness of recombinant proteins. These results were also confirmed by immunofluorescence study. In addition, cytoplasmic regions of ER close to the plasma membrane seemed to be more stained with the anti-SERCA2c antibody (results not shown).

Comparative functional characteristics of the human SERCA2c recombinant protein

The comparative Ca^{2+} pumping properties of the recombinant SERCA2 proteins were studied by using various techniques (Figures 2 and 3). These included studies of Ca^{2+} mobilization in intact cells (Figure 2) and analyses of the SERCA2 catalytic cycle (Figure 3) using microsomes.

Studies in intact cells

First, in our work with intact cells, the effect of overexpression of SERCA2c on intracellular $[Ca^{2+}]$ was studied and compared with that of SERCA2a and SERCA2b (Figure 2). To determine the resting cytosolic Ca^{2+} concentration ($[Ca^{2+}]_C$) and that obtained

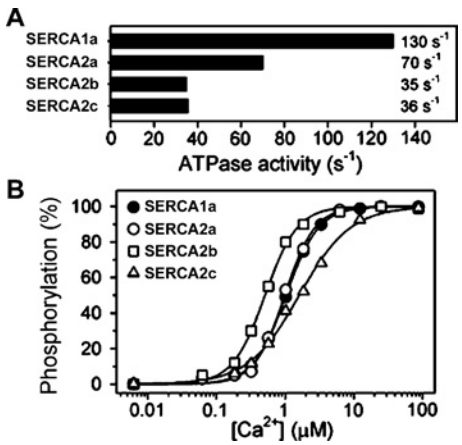


Figure 3 Recombinant SERCA2c protein shows similar turnover rate to SERCA2b but a lower affinity for Ca^{2+}

(**A**) The ATPase turnover rates were determined as described in [23]. Bars represent the ATPase activities of SERCA2a (70 s^{-1}), SERCA2b (35 s^{-1}) and SERCA2c (36 s^{-1}) compared with SERCA1a (130 s^{-1}) ($n=3$). (**B**) Ca^{2+} affinity for activation of phosphorylation from ATP. Phosphorylation was carried out in the presence of 5 μM [γ-^{32}P]ATP and the indicated concentrations of free Ca^{2+} [23]. The data were normalized by taking as 100 % the maximum phosphorylation reached, and the 'lines' show the best fits of the Hill equation, giving the following $K_{0.5}$ values: SERCA1a, 1.030; SERCA2a, 0.985; SERCA2b, 0.508; and SERCA2c, 1.604 ($n=3$ or 4).

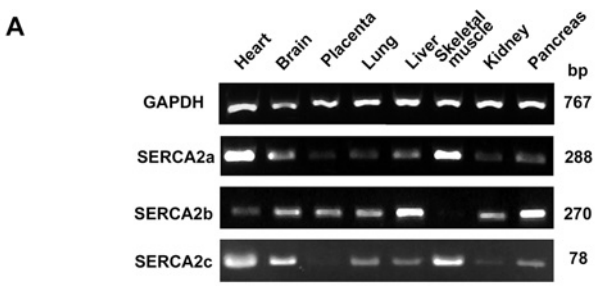
after treatment with the Ca^{2+} ionophore ionomycin, SERCA2-transfected cells were loaded with the Ca^{2+} indicator fura 2/AM. The Ca^{2+} response induced by ionomycin in the presence of EGTA (to prevent Ca^{2+} influx from extracellular space) was used as an estimate of the Ca^{2+} content of the ER ($[Ca^{2+}]_{ER}$) [9,11]. Figure 2(A) shows typical measurements of cytosolic $[Ca^{2+}]$ using empty vector pcDNA3.1- and SERCA2-transfected cells, at resting state, in the presence of EGTA ($[Ca^{2+}]_C$) and after addition of ionomycin ($[Ca^{2+}]_{ER}$). In Figure 2(B), the quantitative comparisons of $[Ca^{2+}]_C$ and $[Ca^{2+}]_{ER}$ are shown. $[Ca^{2+}]_C$ of HEK-293 cells transfected with pcDNA3.1, SERCA2a, SERCA2b and SERCA2c constructs were found to reach 55 ± 3, 63 ± 4, 50 ± 1 and 53 ± 4 nM respectively. The $[Ca^{2+}]_{ER}$ in the same cells was found to reach 191 ± 8, 583 ± 21, 559 ± 14 and 698 ± 23 nM respectively. Hence, SERCA2c protein shows similar effects on both $[Ca^{2+}]_C$ and $[Ca^{2+}]_{ER}$ as SERCA2a and SERCA2b proteins; in fact $[Ca^{2+}]_{ER}$ was slightly higher for SERCA2c.

Studies of isolated membrane proteins

Secondly, we examined the catalytic cycle of SERCA2c recombinant protein (Figure 3) and compared the results with the well-characterized SERCA1a, SERCA2a and SERCA2b isoforms [14,23]. For this, we used microsomes isolated from the stably transfected HEK-293 cells. Figure 3(A) shows that the turnover rate for ATP hydrolysis of SERCA2c was 36 s^{-1}, which is quite similar to the reported value [14] for SERCA2b (35 s^{-1}), but lower than the reported values [14] for SERCA2a (70 s^{-1}) and SERCA1a (130 s^{-1}). The Ca^{2+} dependence of phosphorylation by [γ-^{32}P]ATP at 0 °C (Figure 3B) gave $K_{0.5}$ values for Ca^{2+} activation of 0.985, 0.508, 1.604 and 1.030 μM for SERCA2a, SERCA2b, SERCA2c and SERCA1a respectively, in line with previous measurements for SERCA1a, SERCA2a and SERCA2b isoforms [14], and demonstrating the lowest Ca^{2+} affinity for SERCA2c. In this connection, it should be noted that the assay for ATP hydrolysis referred to above was carried out under conditions where Ca^{2+} activation of ATP hydrolysis was at maximum even for SERCA2c. Hence, SERCA2c protein has unique properties, being characterized by a lower apparent affinity for cytosolic Ca^{2+} than SERCA2a and SERCA2b proteins and by a SERCA2b-like maximal catalytic turnover rate.

Human heart expresses SERCA2c mRNA

To explore the tissue distribution pattern, the expression of the SERCA2c mRNA was monitored by PCR in a panel of commercially available human tissue cDNAs (Clontech). In contrast with the ubiquitous expression of SERCA2b, SERCA2c displays a SERCA2a-like mRNA distribution (Figure 4). SERCA2c mRNA was rather high in the heart (100 %) and skeletal muscle (67.6 % versus heart). SERCA2c mRNA was also found in other tissues,



B

	Heart	Brain	Placenta	Lung	Liver	Sk.muscle	Kidney	Pancreas
SERCA2a/GAPDH	100	52.6±10.7	9±4.2	16.1±7.6	24±7.3	79.7±14.3	13.4±5.5	20.2±5.1
SERCA2b/GAPDH	18.8±0.6	58.8±5.0	53.8±5.7	74.9±5.9	100	3.3±0.8	51.4±2.9	110.3±7.9
SERCA2c/GAPDH	100	38.7±12.3	6.7±2.1	10.5±4.7	12±1.4	67.6±11.2	13.6±6.8	15.8±4.4

Figure 4 Differential distribution of SERCA2c mRNA in human tissues

(**A**) PCRs showing the expression of SERCA2a, SERCA2b, SERCA2c and GAPDH transcripts in the indicated tissues [9,11] were performed using 1.25 ng of normalized cDNAs (Clontech) and the primers indicated in Supplementary Table 1 (at http://www.BiochemJ.org/bj/395/bj3950249add.htm) for 19 cycles (SERCA2a and SERCA2b) and 26 cycles (SERCA2c and GAPDH). (**B**) For quantifications, the values of heart (normalized to GAPDH) were arbitrarily taken as 100 % for SERCA2a, SERCA2c and GAPDH or those of liver for SERCA2b. The expressions are given as percentages of heart or liver values (means ± S.E.M. for $n=6$).

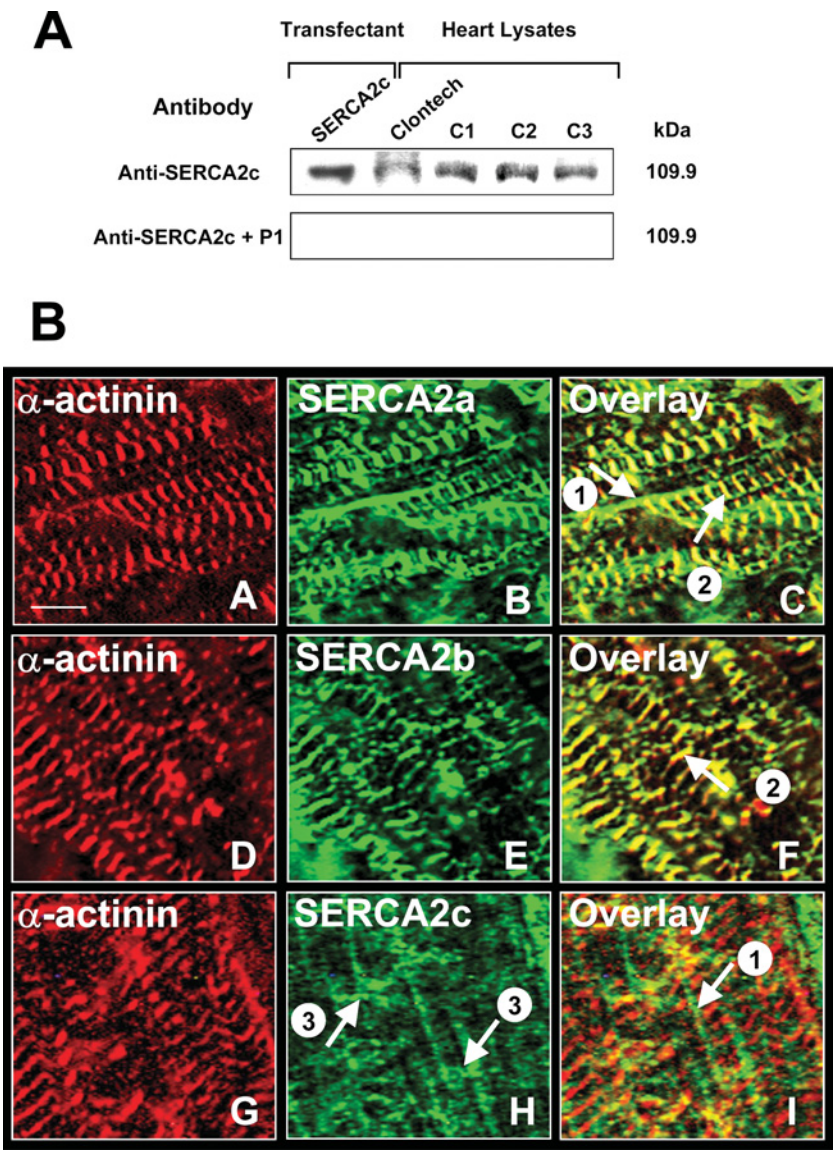


Figure 5 SERCA2c is endogenously expressed in the human heart in a more restricted area than SERCA2a and SERCA2b proteins

(**A**) Recombinant SERCA2c-containing microsomes (10 μg), total human heart protein lysate from Clontech (200 μg) and protein lysates (C1–C3) isolated from the left ventricle of three non-failing hearts (30 μg) were analysed by Western blotting using the anti-SERCA2c antibody in the absence (anti-SERCA2c) or presence (anti-SERCA2c+P1) of 10 μM peptide (P1) used for immunization ($n = 3$). (**B**) Co-immunolabelling of α-actinin and SERCA2 proteins in left ventricle sections from human heart. Overview of fixed heart tissue, double stained with anti-α-actinin (A, D and G) and anti-SERCA2a (B), -SERCA2b (E) or -SERCA2c (H) antibodies. C, F and I show merged images of stainings for α-actinin and SERCA2 proteins. Arrows 1, 2 and 3 indicate longitudinal SR, transversal SR and intercalated discs respectively. The scale bar represents 2 μm ($n = 3$).

but at lower values, varying from 6.7 % in placenta and 15.8 % in pancreas to 38.7 % in brain. In conclusion, SERCA2c mRNA appeared to be mainly expressed in cardiac- and skeletal-muscle tissues.

SERCA2c protein is endogenously expressed in human heart and located in a more restricted area than SERCA2a and SERCA2b proteins

To prove the existence of endogenously expressed SERCA2c protein (Figure 5), immunoblotting studies were first performed (Figure 5A) using our SERCA2c-specific antibody and a sample of total heart lysates (commercially available) as well as protein lysates isolated from left ventricles of three different normal hearts. The SERCA2c protein was detected in different samples and migrated with the expected electrophoretic mobility exactly as the recombinant SERCA2c protein. The SERCA2c-specific immunostaining signal could be abolished in the presence of 10 μM antigenic P1 peptide.

To test whether SERCA2c protein is also expressed specifically in cardiomyocytes, we performed comparative immunocytochemistry experiments to localize the human SERCA2 proteins in fixed left ventricle tissue. Figure 5(B) shows typical images of immunostained longitudinal sections. Heart tissues were doubly stained with a monoclonal anti-α-actinin antibody to delineate the Z-bands and with polyclonal anti-SERCA2 antibodies. For the anti-SERCA2b and -SERCA2c antibodies, due to the expected lower expression of the SERCA2b and SERCA2c proteins compared with that of SERCA2a protein (note the number of PCR cycles in Figure 4), a technique of

staining amplification was used. Characteristic striated staining patterns of fixed cardiac tissues were observed for SERCA2a and SERCA2b proteins in agreement with earlier reports [25–28], namely the similar subcompartmental localization and uniform distribution of SERCA2a and SERCA2b proteins in the SR. However, SERCA2a protein appeared to be targeted to regions close to the T-tubules (transverse immunostaining) as well as to longitudinal SR (longitudinal immunostaining), while SERCA2b protein appeared to be targeted to regions close to the T-tubules only (transversal immunostaining). In contrast, SERCA2c protein was found to present a distinct staining associated only with the longitudinal and intercalated discs in close proximity to the sarcolemma. This points to a confined localization of SERCA2c protein in the subsarcolemmal region of myocardial cells.

Comparative expression of SERCA and PMCA mRNAs and proteins in normal and failing hearts

Taken together, the above functional results (Figures 2 and 3) suggest that the expressed SERCA2c isoform (Figure 5) may participate in the regulation of Ca^{2+} signalling in the heart tissue. In addition, we investigated the expression of the PMCA isoforms. Compared with SERCAs, very little is known about PMCAs in the human heart, especially at protein level. However, PMCA1b and PMCA4b, the known 'housekeeping' isoforms, are also found in adult human heart. Other PMCA1a, PMCA1c, PMCA1d and PMCA4a mRNAs have also been described [29–31]. In the present study, we demonstrated the co-expression of PMCA1c, PMCA1b, PMCA4a and PMCA4b mRNAs, among which, the 'b' mRNA variants presented the highest expression level in various regions of the human heart. The identity of the PMCA1c- and PMCA4a-derived cDNA fragments was confirmed by their sequencing. The obtained sequences were 100 % identical with the corresponding sequences documented in [32] (see Supplementary Figure 1 at http://www.BiochemJ.org/bj/395/bj3950249add.htm). Figures 6 (typical results) and 7 (statistical analysis) show comparative mRNA and protein expression levels of the indicated Ca^{2+}-ATPase isoforms, in four left ventricles isolated from normal donors (C1–C4) and five left ventricles isolated from failing hearts (P1–P5) obtained from patients presenting mixed cardiomyopathy (patient P1) and idiopathic dilated cardiomyopathies (patients P2–P5). Figures 6(A) and 7(A) show that, relative to normal donors, which exhibited insignificant variations in SERCA and PMCA mRNAs, significant modulations of the expressions of SERCA and PMCA mRNAs were found in the failing hearts. A decreased expression of SERCA2a mRNA was observed in all patients, in agreement with the literature data [33–37]. SERCA2b and SERCA2c mRNAs showed modest variations in their expressions with the exception that the SERCA2c mRNA level was slightly increased in patient P1. For PMCAs, almost all patients were found to express higher levels of PMCA1c, thought previously to be a minor splice variant. The mRNA level for another minor splice variant, PMCA4a was also up-regulated in patients P3–P5. The normally highly expressed PMCA4b mRNA showed only a modest increased expression in two patients (P1 and P2). For SERCA and PMCA proteins (Figures 6B and 7B), SERCA2 and PMCA proteins were visualized by using the IID8 and 5F10 monoclonal antibodies respectively. The SERCA2a and SERCA2c proteins were visualized by using the SERCA2-specific polyclonal antibodies. Monoclonal antibody JA3 was specifically used for the PMCA4b immunostaining. In agreement with mRNA data, insignificant variations in expression profiles of the different SERCA and PMCA isoforms were observed in normal donors. In failing hearts, the SERCA2 proteins were found to be slightly decreased in patients P3–P5, while the PMCAs did show an increased expression in all the patients examined. The mean ratios of all PMCA isoforms versus all SERCA2 isoforms reached 1.5 ± 0.5, 1.8 ± 0.3, 2.4 ± 0.8, 3.1 ± 0.9 and 3.8 ± 1.4 in patients P1–P5 respectively. The SERCA2a protein was found to present modest down-regulations varying from 12 ± 6 to 43 ± 5 %. The SERCA2c protein exhibited a significantly higher expression level in patient P1 (166 ± 26 %). An increased expression of PMCA4b was found in patients P1 and P2 (190 ± 5 and 143 ± 26 % respectively). Taken together, the results of mRNA and protein studies support each other and agreed with (i) the down-regulation of SERCA2a protein in both mixed cardiomyopathy and idiopathic dilated cardiomyopathy, (ii) the up-regulation of SERCA2c protein in mixed cardiomyopathy and (iii) the up-regulation of PMCA proteins in both mixed cardiomyopathy and idiopathic dilated cardiomyopathy.

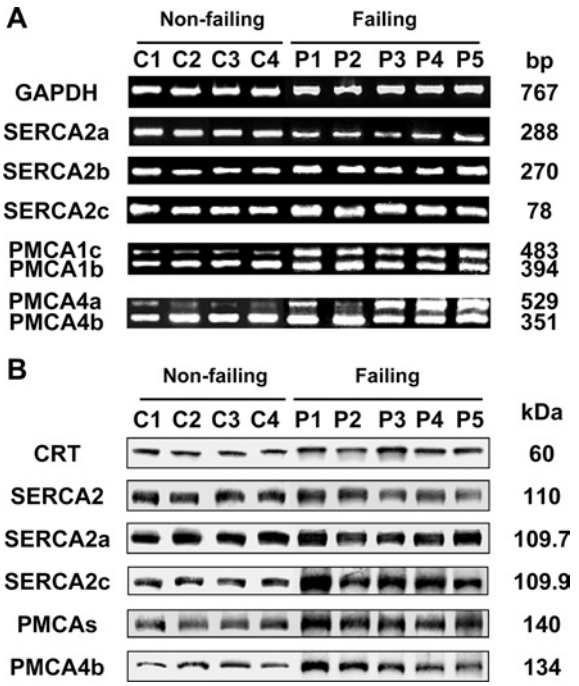


Figure 6 mRNA and protein expression levels of SERCA2 and PMCAs in normal and failing hearts (representative experiments)

(**A**) RNA study. The RT–PCR experiment shows the expression of GAPDH (after 19 PCR cycles), SERCA2a (after 16 PCR cycles), SERCA2b (after 19 PCR cycles), SERCA2c and PMCA1c–PMCA1b (after 26 PCR cycles) and PMCA4a–PMCA4b (after 27 PCR cycles) mRNAs in hearts isolated from four normal subjects (C1–C4) and five patients with cardiomyopathies (P1–P5), mixed cardiomyopathy for patient P1 and idiopathic dilated cardiomyopathy for patients P2–P5 using the primers indicated in Supplementary Table 1 (at http://www.BiochemJ.org/bj/395/bj3950249add.htm). (**B**) Protein study. Western blots of calreticulin (CRT), total SERCA2, SERCA2a, SERCA2c, total PMCAs and PMCA4b proteins using 30 μg of left ventricle lysate proteins and the corresponding anti-calreticulin, IID8, anti-SERCA2a, anti-SERCA2c, 5F10 and JA3 antibodies respectively.

DISCUSSION

Intracellular Ca^{2+} handling is the central co-ordinator of cardiac contraction and relaxation [33]. The regulation of cytosolic Ca^{2+} within cardiac myocytes is dependent on the integration of several carefully orchestrated mechanisms. Traditionally, upon excitation, Ca^{2+} is released from an internal storage pool in the SR through a Ca^{2+} release channel (ryanodine receptor channel). This causes an increase in cytosolic Ca^{2+}, triggering systolic

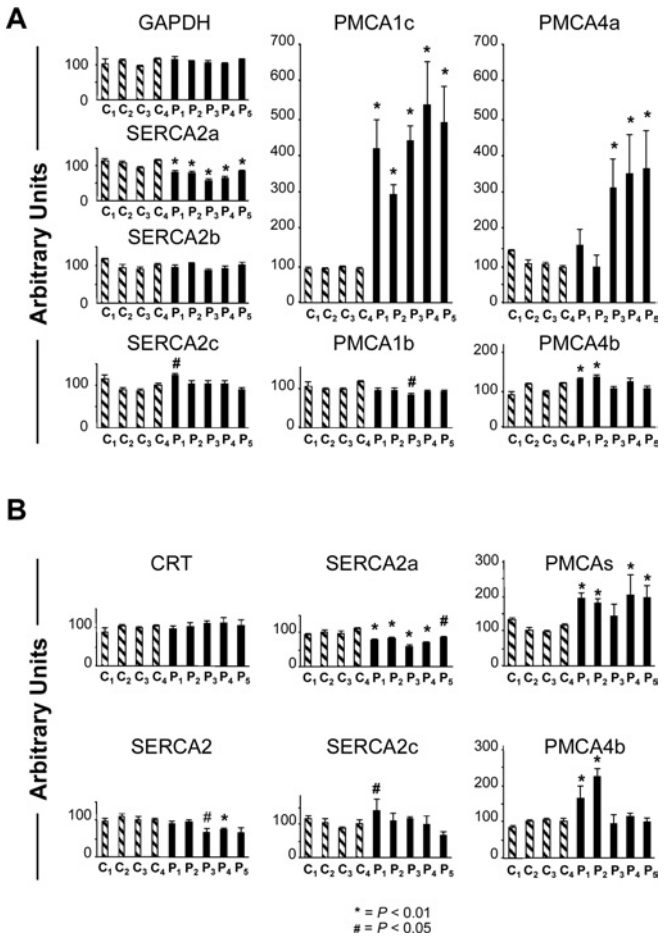


Figure 7 Comparative expression of SERCA2 and PMCA mRNAs and proteins in normal (C1–C4) and failing hearts (P_1–P_5) (summary of densitometric data)

(**A**) RNA study. For quantifications of non-failing and failing hearts, the mean value of the different normal hearts was arbitrarily taken as 100 %. For non-failing hearts, results are expressed as the means ± S.E.M. for $n = 5$. For quantifications of failing hearts, results were corrected for GAPDH and are expressed as the means ± S.E.M. for $n = 9$–15. $^{*}P < 0.01$ and $^{\#}P < 0.05$ compared with control. (**B**) Protein study. For quantifications of non-failing and failing hearts, the mean value of the different non-failing hearts was arbitrarily taken as 100 %. For quantifications of failing hearts, results were corrected for calreticulin and are expressed as the means ± S.E.M. for $n = 5$–9. $^{*}P < 0.01$ and $^{\#}P < 0.05$ compared with control.

contraction. During diastole, cardiac relaxation is subsequently initiated by Ca^{2+} removal from the cytosol. This is partly due to the activity of SERCAs and PMCAs.

Non-failing human heart appears to be equipped with a total of at least seven SERCA and PMCA isoforms including SERCA2a, SERCA2b, SERCA2c, PMCA1c, PMCA1b, PMCA4a and PMCA4b. The co-expression of both cardiac SERCA2a isoform and housekeeping SERCA2b isoform in heart tissue has been known for a while. In the present study, we show for the first time that the third human SERCA2 isoform, SERCA2c, is also expressed in the cardiac tissue. Recombinant SERCA2c protein expressed in transfected HEK-293 cells was found to be functionally active. Further investigation of the catalytic parameters demonstrated that SERCA2c exhibits functional similarities, but also functional differences relative to SERCA2a and SERCA2b proteins. Thanks to a newly developed anti-SERCA2c antibody, the endogenous SERCA2c protein was found to be co-expressed with SERCA2a and SERCA2b in human heart lysate. Its distribution in human cardiomyocytes points to a unique localization compared with those of SERCA2a and SERCA2b proteins. Similar distributions of SERCA2a and SERCA2b isoforms have already been reported in transgenic mice overexpressing SERCA2b in heart [27] or mice in which the SERCA2a protein is replaced by the SERCA2b isoform [28]. The Ca^{2+} concentration near the plasma membrane is very high compared with the rest of the cytosol. The lower apparent affinity for cytosolic Ca^{2+} displayed by SERCA2c relative to both SERCA2a and SERCA2b proteins may actually represent a functional adaptation of the SERCA2c specifically tuned to work in an environment eliciting local high $[Ca^{2+}]$. The specific C-terminus of SERCA2c must be responsible for the observed lower apparent affinity for Ca^{2+}. In addition, based on the fact that SERCA2a and SERCA2c present relatively similar lengths of their alternatively spliced C-terminus (Figure 1B), one can suggest that the SERCA2c carboxyl tail may be involved in the restricted targeting of SERCA2c in a subplasmalemmal area of both HEK-293 cells and cardiomyocytes. At this moment, it is not clear why SERCA2c displays an approx. 2-fold decrease in its apparent affinity for Ca^{2+} relative to SERCA2a (cf. Figure 3B) and whether or not this difference in the Ca^{2+} affinity has immediate physiological consequences. Concerning PMCA isoforms in human heart, only one report described evidence for the degradation products derived from PMCA4a and PMCA4b proteins [21]. In the present study, we confirmed the co-expression of PMCA1c–PMCA1b and PMCA4a–PMCA4b mRNAs in human heart and showed the expression of intact PMCA4b isoform.

Heart failure is associated with an abnormal Ca^{2+} handling. As a consequence, early studies have shown that both the cardiac SERCA2a protein and its activity are reduced in diseased cardiac muscle [34–37], although some controversial results are also known [18,19,38,39]. The reasons for these discrepancies are not clear. Some differences could be due to the studies of either total SERCA2 or only SERCA2a mRNAs and proteins and/or to different functions of the various Ca^{2+}-ATPase isoforms according to the cardiomyopathies. In this context, we investigated the expression of the various members of SERCA and PMCA families in some patients with cardiomyopathies. An up-regulation of the expression of SERCA2c protein has been observed in the patient presenting a mixed cardiomyopathy. This would support a role of SERCA2c protein in human heart pathophysiology. We also found a modulation of the expression of SERCA2c during monocytic differentiation [6]. In addition, in almost all patients, the levels of PMCA1c and PMCA4a mRNAs were increased. Similar observations were found at mRNA level when studying patients with dilated cardiomyopathy or coronary artery disease (results not shown). So, this would appear as a general feature of cardiomyopathies. These changes in the expression levels appeared to be also accompanied by an up-regulation in the level of PMCA4b protein in two patients with mixed and idiopathic cardiomyopathies.

Overexpression of PMCA4 in the myocardium of transgenic rats [40] failed to significantly alter cardiac function or resting $[Ca^{2+}]_C$, suggesting that interdependent Ca^{2+} signalling systems function to maintain $[Ca^{2+}]$. Meanwhile, progress has been made in the study of PMCA4a and PMCA4b proteins. PMCA4a and PMCA4b arise from the alternative splicing (3′-end) of an exon, which is either included (in PMCA4a) or excluded (in PMCA4b). Inclusion of this exon changes the C-terminal part of the protein, thus generating a calmodulin (activator of PMCAs)-binding domain. A feature of the C-terminal end is an inhibitory region, which is located downstream of the calmodulin-binding domain of PMCA4b, but it is not present in PMCA4a [41]. Therefore the increased expression of PMCA4a may be related

to the existence of higher than average Ca^{2+} levels in cardiomyopathies. More recently, the C-terminal of the 'b' splice variants has been shown to interact with members of the MAGUK (membrane-associated guanylate kinase) family of PDZ domain-containing proteins, including nNOS (neuronal nitric oxide synthase) [42]. PMCA and nNOS localize in caveolae in cardiomyocytes [40]. An increased nNOS-derived nitric oxide production has been shown in some cardiomyopathies [43]. Hence, the increase in PMCA4b expression may be involved in the regulation of the activity of nNOS by reducing the local Ca^{2+} concentration, leading to reduced activity of nNOS, a calcium/calmodulin-dependent isoform of nitric oxide synthase.

In conclusion, the present study shows that human heart expresses a number of Ca^{2+}-ATPase isoforms including the novel SERCA2c protein. In addition, the study of SERCA and PMCA expression in failing hearts from patients with cardiomyopathies suggests that SERCAs and PMCAs involved in Ca^{2+} homoeostasis may participate in the regulation of heart function. It appears that various and intricate compensatory processes are active *in vivo* in restoring intracellular $[Ca^{2+}]$.

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