Gene Expression of the Cardiac Na\(^+\)-Ca\(^{2+}\) Exchanger in End-Stage Human Heart Failure

Roland Studer, Hans Reinecke, Johannes Bilger, Thomas Eschenhagen, Michael Böhml, Gerd Hasenfuß, Hanjörg Just, Jürgen Holtz, Helmut Drexler

Abstract The regulation of cytosolic Ca\(^{2+}\) concentration during excitation-contraction coupling is altered in the failing human heart. Previous studies have focused on disturbances in Ca\(^{2+}\) release and reuptake from the sarcoplasmic reticulum (SR), whereas functional studies of the cardiac Na\(^+\)-Ca\(^{2+}\) exchanger, another important determinant of myocyte homeostasis, are lacking for the failing human heart. Using a cardiac Na\(^+\)-Ca\(^{2+}\) exchanger cDNA recently cloned from a guinea pig cDNA library, we investigated the gene expression of the cardiac Na\(^+\)-Ca\(^{2+}\) exchanger in relation to the SR Ca\(^{2+}\)-ATPase. Expression of both genes was quantified in left ventricular myocardium from 24 failing human cardiac explants and 7 control heart samples in relation to β-myosin heavy chain mRNA by slot blot analysis. Compared with patients with nonfailing hearts, patients with dilated cardiomyopathy (DCM, n=13) showed a 55% increase in Na\(^+\)-Ca\(^{2+}\) exchanger mRNA levels (P<.05 versus control value) and a 41% increase in patients with coronary artery disease (CAD, n=11). In the same hearts, SR Ca\(^{2+}\)-ATPase mRNA levels were decreased by 50% in DCM and by 45% in CAD (P<.05 for both versus control value). There was a positive correlation between Na\(^+\)-Ca\(^{2+}\) exchanger and SR Ca\(^{2+}\)-ATPase mRNA levels both in normal and failing human hearts, albeit with different slopes and intercepts of the regression line. The Na\(^+\)-Ca\(^{2+}\) exchanger protein levels as assessed by Western blot analysis and normalized to β-myosin heavy chain protein were increased in DCM and CAD (P<.05 and P<.01 versus control value, respectively), whereas SR Ca\(^{2+}\)-ATPase protein levels were reduced (P<.05 for both groups versus control values). Thus, the Na\(^+\)-Ca\(^{2+}\) exchanger gene expression is enhanced in failing human hearts and may, in part, compensate for the depressed SR function with regard to diastolic Ca\(^{2+}\) removal. (Circ Res. 1994;75:443-453.)

Key Words • dilated cardiomyopathy • coronary artery disease • Ca\(^{2+}\) homeostasis • myocardial gene expression

Intracellular Ca\(^{2+}\) homeostasis of myocytes is maintained by Ca\(^{2+}\) release and uptake by the sarcoplasmic reticulum (SR)\(^1\) and the Ca\(^{2+}\) flux across the sarcolemma.\(^2,3\) The Ca\(^{2+}\) release out of the SR occurs via the ryanodine receptor (Ca\(^{2+}\) release channel) and is largely responsible for the tension development of myocardium.\(^1\) Myocardial relaxation depends on the function of ATP-supported SR Ca\(^{2+}\)-ATPase, which mediates the reuptake of Ca\(^{2+}\) into the SR after each systole.\(^1\) Although there are contradictory results,\(^4\) several studies focusing on disturbances in Ca\(^{2+}\) reuptake from the SR demonstrated a reduction of SR Ca\(^{2+}\)-ATPase gene expression, SR Ca\(^{2+}\)-ATPase protein levels, and in vitro Ca\(^{2+}\) accumulation into the SR in failing hearts.\(^5-8\) The decrease in density of the Ca\(^{2+}\) pumps, which leads to a slower rate of Ca\(^{2+}\) uptake into the SR, may, in part, account for prolongation of the Ca\(^{2+}\) transients,\(^9,10\) impaired isometric relaxation, and reduced tension-independent heat production observed both in experimental and human heart failure.\(^11,12\) However, although ≈80% of the Ca\(^{2+}\) flux involved in excitation-contraction coupling occurs across the SR membrane, 20% of the Ca\(^{2+}\) is moved across the sarcolemma by two transport systems, ie, the Na\(^+\)-Ca\(^{2+}\) exchanger and the plasma membrane Ca\(^{2+}\) pump.\(^13,14\) Bridge et al\(^15\) recently showed the ability of the exchanger to extrude Ca\(^{2+}\) from guinea pig ventricular myocytes to produce relaxation. These studies indicate that Na\(^+\)-Ca\(^{2+}\) exchange is the dominant cardiac Ca\(^{2+}\) efflux mechanism but that there is little contribution of the plasma membrane Ca\(^{2+}\) pump.\(^13-15\) The Na\(^+\)-Ca\(^{2+}\) exchanger uses the influx of Na\(^+\) into the cell to extrude Ca\(^{2+}\) in a ratio of three Na\(^+\) to one Ca\(^{2+}\), thereby generating an inward current. However, in the initial phase of excitation-contraction coupling, Na\(^+\)-Ca\(^{2+}\) exchange may occur in the opposite direction. In this respect, recent studies have implicated a putative role for the Na\(^+\)-Ca\(^{2+}\) exchanger in the activation of contraction by providing a source of Ca\(^{2+}\) to trigger the release of additional Ca\(^{2+}\) from cardiac SR.\(^16\)

Although the significance of Na\(^+\)-Ca\(^{2+}\) exchange in cardiac excitation-contraction coupling is now being recognized, the status of the Na\(^+\)-Ca\(^{2+}\) exchanger in heart failure remains controversial. Functional data obtained by analyzing kinetics of Na\(^+\) accumulation in membrane vesicles are subject to methodological criticism\(^2\) and have demonstrated contradictory results in animal models of cardiac overload.\(^17,18\) However, the recent purification\(^21\) and molecular cloning\(^22\) of the canine cardiac sarcolemmal Na\(^+\)-Ca\(^{2+}\) exchanger protein have provided a powerful means to investigate the expression and regulation of the Na\(^+\)-Ca\(^{2+}\) exchanger in various pathological conditions at the molecular level. Preliminary data from pooled human hearts did not reveal significant changes of Na\(^+\)-Ca\(^{2+}\) exchanger
mRNA levels in failing hearts. In the present study, we examined the gene expression and protein level of the Na\(^+\)-Ca\(^{2+}\) exchanger in a large series of well-defined failing human hearts compared with nonfailing human hearts and their relation to the expression of SR Ca\(^{2+}\)-ATPase.

**Materials and Methods**

**Patients**

Failing human hearts were obtained from patients undergoing heart transplantation, including 13 patients with dilated cardiomyopathy and 11 patients with coronary artery disease. All patients were in New York Heart Association functional class IV heart failure and demonstrated abnormal pretransplant hemodynamics (see Table 1). Between the two groups of patients, there were no significant differences in mean age, mean cardiac index, mean pulmonary capillary wedge pressure, or mean left ventricular ejection fraction. Nonfailing control hearts (see Table 2) were obtained from organ donors whose hearts could not be used for transplantation because of surgical reasons or because of blood group incompatibility. None of the organ donors had a history of heart failure, and their mean age did not differ significantly from patients with heart failure. Tissue samples of the left ventricular free wall were taken at the time of explantation, immediately frozen in liquid nitrogen, and stored at \(-80^\circ\text{C}\) until use. Care was taken not to take scarred, fibrotic, or adipose tissue, endocardium, epicardium, or great vessels. The present study was approved by the ethical committee of the University of Freiburg.

**RNA Preparation**

Membrane was removed from \(\approx200\) mg of frozen left ventricular tissue with a Mikro-Dismembrator II (B. Braun Melsungen Inc). Total cellular RNA was isolated from the frozen tissue by the method of acid guanidinium thiocyanate/phenol/chloroform extraction and stored in diethyl pyrocarbonate–treated water at \(-20^\circ\text{C}\). The integrity of the RNA was
checked by agarose gel electrophoresis and ethidium bromide staining. RNA concentration was evaluated in triplicate by absorbance at 260 nm by use of a DU-65 spectrophotometer (Beckman Instruments Ltd).

**Protein Preparation**

Membrane was removed from ≈100 mg of frozen left ventricular tissue with a Mikro-Dismembrator II (B. Braun Melsungen Inc) for 10 seconds, homogenized in a sevenfold volume of lysis buffer (mmol/L: HEPES 20, EGTA 4, and diithiothreitol 1, pH 7.5) completed with protease inhibitors (mmol/L: leupeptin 0.1 and phenylmethylsulfonyl fluoride 0.3 (Sigma Ltd), and stirred for 45 minutes at 4°C. After centrifugation at 100 000g for 60 minutes at 4°C in an L-5-65 ultracentrifuge (Beckman Instruments Ltd), the supernatant was carefully removed, and the pellet containing the particulate fraction was resuspended in a sevenfold volume of lysis buffer. The protein concentration of the soluble and particulate fraction was determined in triplicate by Coomassie blue staining using ovalbumin as a standard according to the Bradford method. Aliquots were stored at −80°C until use.

**Northern Blot Analysis**

Total RNA (10 μg) was prepared and subjected to electrophoresis according to Rosen et al., transferred to a nylon membrane (Amersham Buchler Ltd) by overnight capillary blotting, and fixed to the membrane by UV irradiation. The blot membrane was successively hybridized with a 1.5-kb guinea pig Na⁺-Ca²⁺ exchange cDNA fragment (*EcoR*I-*EcoR*I) (K.D. Philipson, unpublished data), a 1.2-kb rat SR Ca²⁺-ATPase cDNA fragment (*EcoR*I-*EcoR*I), a 1.6-kb human β-myosin heavy chain (β-MHC) cDNA fragment (*Neo I*-HindIII), a 0.58-kb rat atrial natriuretic factor (ANF) cDNA fragment (*Pst*I-*Pst*I), which is 85% identical to the human ANF cDNA sequence, and an oligonucleotide (5'-GGTATCTCTGATCTTTGAA-3') specific to the rat 18S rRNA. The Na⁺-Ca²⁺ exchange cDNA fragment contains ≈1200 bp of coding and ≈300 bp of noncoding sequences of the 3' end of a full-length cDNA clone that has been isolated from a guinea pig heart λ-Zap primary cDNA library. The homology of the total guinea pig Na⁺-Ca²⁺ exchange cDNA sequence as well as the cDNA fragment used for hybridization is at least 90% compared with those cDNA sequences of dogs, humans, and rats and was found to be expressed as a single band at 7 kb in Northern blot analysis of rat heart tissue. In addition, the full-length cDNA clone of the guinea pig heart Na⁺-Ca²⁺ exchange has been functionally expressed in oocytes (K.D. Philipson, personal communication).

obtain radioactive labeled cDNA probes, the inserts were cut out from the plasmid vectors by using the appropriate restriction enzymes and separated from the vector DNA on a low-melting-point sea plaque agarose gel (Biozym). The cDNA fragments were labeled with [α-³²P]dCTP (Amersham Buchler Ltd) by using a Multiprime DNA labeling kit according to the supplier’s recommendation (Amersham Buchler Ltd). The 5' end labeling of the 18S rRNA oligonucleotide with [α-³²P]dATP (Amersham Buchler Ltd) was performed with T4 kinase (Amersham Buchler Ltd) as previously described by Schäfer et al. For cDNA hybridization, the blot membrane was prehybridized for 4 hours at 65°C in hybridization buffer containing 2x standard saline citrate (SSC), containing 0.3 mol/L NaCl and 0.03 mol/L sodium citrate, pH 7.0, 10× Denhardt’s solution (0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, and 0.2% Ficoll), 0.1% sodium dodecyl sulfate (SDS), 0.1% pyrophosphate, 2 mmol/L EDTA, 2.5% dextran sulfate, and 50 μg/mL sonicated denatured *Escherichia coli* DNA (Sigma Ltd). Hybridization was performed in fresh hybridization solution at 65°C for at least 16 hours and completed with the respective radioactive labeled cDNA probe to a concentration of ≈1×10⁶ cpm/mL. After hybridization with the cDNAs of Na⁺-Ca²⁺ exchange, SR Ca²⁺-ATPase, and ANF, the membrane was successively washed in 2× SSC/0.1% SDS, 1× SSC/0.1% SDS, and 0.5× SSC/0.1% SDS for 30 minutes at 60°C, respectively. After β-MHC cDNA hybridization, the washing procedure was as follows: 2× SSC/0.1% SDS, 0.5× SSC/0.1% SDS, and 0.1× SSC/0.1% SDS for 30 minutes at 65°C, respectively. For 18S rRNA oligonucleotide hybridization, the membrane was prehybridized for 1 hour at 49°C in hybridization buffer containing 5× SSPE (0.75 mol/L NaCl, 50 mmol/L Na₂HPO₄, and 5 mmol/L EDTA, pH 7.0), 5× Denhardt’s solution, 0.1% SDS, and 10 μg/mL sonicated denatured *E coli* DNA (Sigma Ltd). Hybridization was done in the same solution completed with the radioactive labeled oligonucleotide in a concentration of 1×10⁶ cpm/mL at 49°C for an additional 4 hours. The hybridization temperature (−5°C) was calculated according to Sugi et al. Membranes were washed three times in 6× SSC at room temperature, followed by a “hot wash” for 1 minute at 49°C in 6× SSC. After the washing procedure, the membranes were kept moist between plastic foils and exposed at −80°C to X-Omat AR x-ray films (Kodak Inc) with Quanta III intensifying screens (Siemens).

**Slot Blot Analysis and Quantification of mRNA**

RNA samples were subjected to a 1:2 dilution (2.5, 5, and 10 μg of total RNA) prepared according to standard protocols and spotted to a nylon membrane (Amersham Buchler

### Table 2. Clinical Characteristics and Cardiac Atrial Natriuretic Factor Gene Expression of Nonfailing Heart Donors

<table>
<thead>
<tr>
<th>Code</th>
<th>Sex</th>
<th>Age, y</th>
<th>Cause of Death</th>
<th>Other Diagnoses</th>
<th>Drugs at HT</th>
<th>ANF mRNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>36</td>
<td>CA, CB</td>
<td>Arteriosclerosis, nicotine abuse</td>
<td>6 mg/h DA</td>
<td>0.04</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>46</td>
<td>CA, CB</td>
<td>Obesity, hypertension, mild arteriosclerosis</td>
<td>6 mg/h DA</td>
<td>0.01</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>56</td>
<td>Polytrauma, CB</td>
<td>Arteriosclerosis</td>
<td>50 mg/h DA</td>
<td>...</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>24</td>
<td>CB</td>
<td>Suspcion of atrial myxoma</td>
<td>None</td>
<td>0.06</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>58</td>
<td>Polytrauma</td>
<td>None</td>
<td>None</td>
<td>0.05</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>52</td>
<td>SAB</td>
<td>None</td>
<td>None</td>
<td>0.01</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>40</td>
<td>CB</td>
<td>None</td>
<td>None</td>
<td>0.01</td>
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<tr>
<td>Mean</td>
<td></td>
<td>44.6</td>
<td></td>
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<tr>
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<td>12.1</td>
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<td>0.02</td>
</tr>
</tbody>
</table>

HT indicates heart transplantation; ANF, atrial natriuretic factor; CA, aneurysm of cerebral artery; CB, intracerebral bleeding; SAB, subarachnoid bleeding; and DA, dopamine.

*ANF mRNA hybridization signal was normalized to the hybridization signal of 18S rRNA.
Western Blot Analysis

Samples of 100 μg protein of particulate or soluble fraction were denatured by heating to 95°C in sample buffer (5% SDS, 13.3% saccharose, 0.083 mol/L dithiothreitol, 0.033 mol/L Tris-HCl, pH 6.8, and 0.0033% bromphenol blue) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 7.5% running gel. Proteins were transferred to a Hybond-ECL nitrocellulose membrane (Amersham Buchler Ltd) by semidry electrobetting (Fast Blot B33, Biometra Ltd) at 5 mA/cm² for 2 hours with 25 mmol/L Tris-HCl, pH 8.3, 150 mmol/L glucose, and 20% methanol as blotting buffer. The transfer was checked by staining the nitrocellulose membrane with 0.1% Ponceau S solution (Sigma Ltd) prepared in 2.5% acetic acid. The blot was blocked overnight in 5% nonfat milk solution (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, and 5% nonfat milk powder) and washed three times for 5 minutes in washing buffer (TBS, containing 20 mmol/L Tris-HCl, pH 7.5, and 150 mmol/L NaCl). For immunoreaction, the blot was incubated with a 1:1000 diluted rabbit anti-dog Na⁺-Ca²⁺ exchanger antisera21 or a 1:3000 diluted mouse anti-dog slow skeletal (and cardiac) Ca²⁺-ATPase monoclonal IgG, antibody solution by semidry electroblotting (2202 Ultrascan laser densitometer, LKB) of the band densities obtained within the linear response range of the x-ray films. For each mRNA species investigated, there was a linear relation in the amount of slot-blotted RNA and the corresponding hybridization signal. RNA levels of 18S rRNA and β-MHC mRNA were used as standards for total cardiac RNA and myocyte-specific RNA, respectively.

Quantification of Immunoreactive Bands

Relative amounts of each protein species were evaluated by densitometric scanning (2202 Ultrascan laser densitometer, LKB) of the band densities obtained within the linear response range of the x-ray films used. Linearity of immunoreaction was checked by detection and quantification of distinct amounts of protein.

Statistical Analysis

All data are presented as mean±SD. Statistical differences between groups were calculated by ANOVA followed by the Student-Newman-Keuls test, the unpaired two-tailed t test, or the Mann-Whitney test as appropriate. Correlations were examined by linear regression analysis. Slope heterogeneities of regression lines were examined by covariance analysis. Significance was accepted at the level of P<.05.

Results

Northern blot analysis of left ventricular myocardium of failing and nonfailing human hearts demonstrated that the cDNA fragment encoding for guinea pig Na⁺-Ca²⁺ exchanger (K.D. Philipson, unpublished data) and rat SR Ca²⁺-ATPase26 hybridized specifically at 7.2 and at 4.3 kb, respectively (Fig 1). Fig 2 shows the expression level of both genes in left ventricular samples of 13 cardiac explants with dilated cardiomyopathy, of 11 cardiac explants with coronary artery disease, and of 7 heart donors without failure, quantified by slot blot hybridization. Compared with nonfailing hearts (0.23±0.14), the gene expression of the Na⁺-Ca²⁺ exchanger normalized to 18S rRNA was significantly increased both in coronary artery disease (0.40±0.22) and in dilated cardiomyopathy (0.43±0.13) without a significant difference between coronary artery disease and dilated cardiomyopathy (Fig 2, top). The gene expression of the SR Ca²⁺-ATPase normalized to 18S rRNA tended to be decreased both in patients with coronary artery disease and in patients with dilated cardiomyopathy; however, the differences did not reach statistical significance compared with nonfailing hearts (0.35±0.23 and 0.28±0.16, respectively, versus 0.49±0.33 for nonfailing hearts) (Fig 2, top).

The ratio of Na⁺-Ca²⁺ exchanger mRNA to β-MHC mRNA was also significantly increased in dilated cardiomyopathy (0.34±0.08 versus 0.22±0.09), indicating that in dilated cardiomyopathy, Na⁺-Ca²⁺ exchanger mRNA is increased not only relative to total cardiac RNA but also to myocyte-specific RNA (Fig 2, bottom). Although the
mean values for Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger mRNA levels normalized to β-MHC mRNA were quite similar in hearts from patients with dilated cardiomyopathy and coronary artery disease, the increase in the ratio of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger mRNA to β-MHC mRNA for the hearts of patients with coronary artery disease did not quite reach statistical significance compared with nonfailing donor hearts (0.31±0.11 versus 0.22±0.09) (Fig 2, bottom). The ratio of SR Ca\textsuperscript{2+}-ATPase mRNA to β-MHC mRNA was significantly decreased in hearts from patients with coronary artery disease (0.23±0.12 versus 0.42±0.17) and from patients with dilated cardiomyopathy (0.21±0.13 versus 0.42±0.17) compared with nonfailing donor hearts (Fig 2, bottom). The divergence in the left ventricular expression of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger and SR Ca\textsuperscript{2+}-ATPase mRNA in the failing human heart is most evident from the markedly increased ratio of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger mRNA to SR Ca\textsuperscript{2+}-ATPase mRNA: 2.38±1.86 in dilated cardiomyopathic hearts and 1.25±0.29 in hearts from patients with coronary artery disease versus 0.55±0.15 in nonfailing donor hearts (P<.05 for hearts from patients with dilated cardiomyopathy versus hearts from patients with coronary artery disease and nonfailing donor hearts); thus, this ratio was significantly higher in patients with dilated cardiomyopathy compared with patients with coronary artery disease. The Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger mRNA...
levels (normalized to β-MHC mRNA) of five patients not previously treated with digoxin (0.36±0.12) were not significantly different from those patients treated with digoxin (0.31±0.09, n=19), suggesting that the increase in Na⁺-Ca²⁺ exchanger mRNA levels is not related to therapy with digoxin.

Both in failing and nonfailing human hearts, there was a significant positive correlation of Na⁺-Ca²⁺ exchanger mRNA levels to SR Ca²⁺-ATPase mRNA levels normalized to 18S rRNA, respectively (Fig 3, top). Similar correlations were observed when the mRNA values of Na⁺-Ca²⁺ exchanger and SR Ca²⁺-ATPase were expressed as a ratio to β-MHC mRNA (Fig 3, bottom). Thus, higher SR Ca²⁺-ATPase gene expression is associated with higher Na⁺-Ca²⁺ exchanger gene expression. However, the regression line for failing hearts was shifted to the right and was associated with a different slope. Thus, for any given SR Ca²⁺-ATPase mRNA level, the Na⁺-Ca²⁺ exchanger mRNA was increased in failing hearts (Fig 3). Therefore, the ratio of Na⁺-Ca²⁺ exchanger to SR Ca²⁺-ATPase mRNA is higher in failing hearts compared with nonfailing hearts.

Western blot analysis using a specific polyclonal antibody directed against canine Na⁺-Ca²⁺ exchanger protein revealed two bands in the particulate protein fraction of nonfailing and failing human hearts, one band of ≈120 kD and another one of ≈40 kD (Fig 4).
No specific immunoreaction was found in the soluble protein fraction of both nonfailing and failing myocardium. The relative density of the 120- and 40-kD band was similar in nonfailing and failing hearts, and the percent increase in the densities of the 120- and 40-kD band was comparable in failing and nonfailing hearts. Therefore, the Na\(^+-\)Ca\(^{2+}\) exchanger protein was quantified by adding up the densities of both immunoreactive bands and expressed in relation to the respective myocardial wet weight, to the corresponding protein content of particulate fraction, and to the relative immunoreactivity of myocyte-specific \(\beta\)-MHC protein (Table 3). As shown in Table 3, whatever mode of normalization was used, the protein level of the Na\(^+-\)Ca\(^{2+}\) exchanger was significantly increased in hearts from patients with coronary artery disease and dilated cardiomyopathy compared with nonfailing donor hearts, whereas there was no significant difference between hearts from patients with dilated cardiomyopathy and coronary artery disease. This is most apparent in Fig 6, which shows Na\(^+-\)Ca\(^{2+}\) exchanger protein levels of failing and nonfailing hearts normalized to \(\beta\)-MHC protein. The yield of total protein and the yield of protein from particulate fraction were comparable in each group investigated and not significantly different between nonfailing and failing human hearts (Table 3). In addition, the relative immunoreactivity of myocyte-specific \(\beta\)-MHC protein was measured by slot blot analysis using a monoclonal antibody specific for human \(\beta\)-MHC protein (Fig 5). Relation to both the myocardial wet weight and the protein content of particulate fraction revealed comparable \(\beta\)-MHC protein levels in each group investigated, with no significant differences between nonfailing and failing hearts (Table 3), suggesting \(\beta\)-MHC as an appropriate myocyte-specific protein standard.

Western blot analysis of SR Ca\(^{2+}\) -ATPase was performed by using a monoclonal antibody raised against canine cardiac SR Ca\(^{2+}\) -ATPase.\(^{35}\) In canine as well as in human cardiac tissue, this monoclonal antibody recognizes specifically a 100-kD band identified as SR Ca\(^{2+}\) -ATPase protein.\(^{37}\) As shown in Fig 4, Western blot analysis of SR Ca\(^{2+}\) -ATPase revealed one band at 100 kD in the particulate protein fraction of nonfailing and failing human hearts. No specific immunoreaction was found in the soluble protein fraction of both nonfailing and failing myocardium. SR Ca\(^{2+}\) -ATPase protein levels obtained by Western blot analysis and related to both the protein content of particulate fraction and the relative immunoreactivity of \(\beta\)-MHC protein were significantly decreased in coronary artery disease as well as in dilated cardiomyopathy (Table 3 and Fig 6). Again, no significant differences were obtained between patients with coronary artery disease and dilated cardiomyopathy. SR Ca\(^{2+}\) -ATPase protein levels related to myocardial wet weight tended to be decreased in hearts from patients with coronary artery disease and from patients with dilated cardiomyopathy; however, the differences did not reach statistical significance compared with nonfailing hearts (Table 3). For each of both protein species, Na\(^+-\)Ca\(^{2+}\) exchanger and SR Ca\(^{2+}\) -ATPase, similar results and even similar levels of significance were obtained if normalization was performed by

### Table 3. Yield of Total Protein, Yield of Protein of Particulate Fraction, and Protein Levels of \(\beta\)-Myosin Heavy Chain, Na\(^+-\)Ca\(^{2+}\) Exchanger, and Sarcoplasmic Reticulum Ca\(^{2+}\) -ATPase Expressed as Relative Immunoreactivity

<table>
<thead>
<tr>
<th></th>
<th>Total Protein per Wet Weight, mg/g</th>
<th>Particulate Fraction Protein per Wet Weight, mg/g</th>
<th>Relative Immunoreactivity per Wet Weight, U/g</th>
<th>Relative Immunoreactivity per Particulate Fraction Protein, U/mg</th>
<th>Relative Immunoreactivity</th>
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<tbody>
<tr>
<td></td>
<td>MHC ((x10^3)) EX SR</td>
<td>MHC ((x10^3)) EX SR</td>
<td>Ex/MHC ((x10^{-4}))</td>
<td>SR/MHC ((x10^{-4}))</td>
<td></td>
</tr>
<tr>
<td>NF ((n=7))</td>
<td>95.0±12.6 65.3±11.0 13.8±3.1 155.3±55.2 219.7±74.9</td>
<td>33.8±10.1 37.5±16.2 52.0±16.7 11.3±3.4 16.4±5.4</td>
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<td></td>
</tr>
<tr>
<td>DCM ((n=13))</td>
<td>110.3±25.5 69.8±20.1 13.9±5.1 275.0±92.9* 171.9±104.5</td>
<td>30.8±11.0 60.9±19.9* 32.4±9.0* 20.5±5.6* 11.6±4.8*</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CAD ((n=11))</td>
<td>117.3±15.7 73.8±13.6 14.3±4.7 312.8±73.51 133.0±63.1</td>
<td>26.6±8.1 59.0±13.3* 27.2±15.11 24.4±10.0† 9.9±4.4†</td>
<td></td>
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</tr>
</tbody>
</table>

MHC indicates \(\beta\)-myosin heavy chain; EX, Na\(^+-\)Ca\(^{2+}\) exchanger; SR, sarcoplasmic reticulum Ca\(^{2+}\) -ATPase; NF, nonfailing control hearts; DCM, hearts from patients with dilated cardiomyopathy; and CAD, hearts from patients with coronary artery disease.

\*P<.05 vs NF; †P<.01 vs NF.
using myocardial content of total protein (data not shown). Notably, there was a significant correlation between SR Ca\(^{2+}\)-ATPase mRNA values normalized to β-MHC mRNA and the corresponding protein values normalized to β-MHC protein \((r = .4920, P < .01, n = 31)\).

As shown in Table 1, most patients demonstrated severely abnormal pretransplant hemodynamics. In addition, SR Ca\(^{2+}\)-ATPase mRNA levels normalized to both 18S RNA and β-MHC mRNA were positively correlated to the cardiac index \((n = 20; r = .6079, P < .01)\) and \(r = .5323, P < .05\), respectively) and ejection fraction \((n = 17; r = .5486, P < .05\) and \(r = .5408, P < .05\), respectively). Furthermore, ANF gene expression was investigated by Northern blot analysis demonstrating a single band at 0.95 kb both in failing and nonfailing heart samples (data not shown). The ANF gene expression was upregulated in failing hearts, whereas the ANF gene expression was very low in nonfailing donor hearts \((1.52 \pm 1.68\) versus \(0.03 \pm 0.02, P < .01;\) see Tables 1 and 2).

**Discussion**

Based on the observation that Na\(^+\)-Ca\(^{2+}\) exchange activity was associated with 70-, 120-, and 160-KD proteins, the molecular cloning, functional expression, and the sequence of the exchange protein have recently been achieved. These studies have shown that the 120-KD protein represents the fully mature exchange protein, whereas a 70-KD protein is likely to be a proteolytic fragment. Subsequently, a predominant and dense distribution of exchange sites in the T tubules of rat and guinea pig myocytes has been demonstrated by immunofluorescence and immunoelectron microscopy, indicating that the Na\(^+\)-Ca\(^{2+}\) exchanger is abundantly located in sarcolemma closest to the release of Ca\(^{2+}\). By using the polyclonal antibody raised against purified canine Na\(^+\)-Ca\(^{2+}\) exchange protein, we have now demonstrated that the Na\(^+\)-Ca\(^{2+}\) exchange protein can be detected in normal and failing human hearts, as suggested by one band corresponding to 120 KD (Fig 4). Similar to the 70-KD protein detected in canine myocardium, the 40-KD protein in the human myocardium is likely to be a proteolytic fragment of the 120-KD Na\(^+\)-Ca\(^{2+}\) exchanger protein (Fig 4). Indeed, when the antibodies bound to the 120-KD protein were eluted from Western blots and again used in Western blot analysis, the 40-KD band was recognized by these eluted antibodies, supporting our contention that this 40-KD protein represents a proteolytic fragment of the Na\(^+\)-Ca\(^{2+}\) exchange protein.

Consistent with Northern blot analysis from canine heart tissue, the Na\(^+\)-Ca\(^{2+}\) exchanger in human myocardium is expressed as a 7.2-kb transcript. Comparison of the Na\(^+\)-Ca\(^{2+}\) exchanger cDNA sequences of dogs, humans, rats, and guinea pigs (K.D. Philipson, unpublished data) shows a high homology (≥90%), indicating a high conservation of the Na\(^+\)-Ca\(^{2+}\) exchanger protein in mammals.

The salient finding of the present study is that the gene expression of the Na\(^+\)-Ca\(^{2+}\) exchanger is increased in failing human hearts, which, in turn, is translated into elevated Na\(^+\)-Ca\(^{2+}\) exchanger protein levels. Vice versa, consistent with previous reports, the SR Ca\(^{2+}\)-ATPase
gene expression was decreased in failing human hearts. \(^3\)\(^,\)\(^3\)\(^9\)\(^,\)\(^4\)\(^0\) In addition, the present study shows that the low SR Ca\(^{2+}\)-ATPase mRNA content is accompanied by a decrease of the SR Ca\(^{2+}\)-ATPase protein levels in these patients; indeed, there was a significant correlation between SR Ca\(^{2+}\)-ATPase mRNA and the corresponding protein values.

For both nonfailing and failing human hearts, we found a positive correlation between SR Ca\(^{2+}\)-ATPase and Na\(^+\)-Ca\(^{2+}\) exchanger mRNA. This supports the notion that the gene expression of Na\(^+\)-Ca\(^{2+}\) exchanger and SR Ca\(^{2+}\)-ATPase is coordinately regulated both in the normal and in the failing human heart. However, the regression line for failing hearts was shifted to the right and was associated with a different slope. Thus, for any given SR Ca\(^{2+}\)-ATPase mRNA level, the Na\(^+\)-Ca\(^{2+}\) exchanger mRNA is increased in failing human hearts. Therefore, the ratio of Na\(^+\)-Ca\(^{2+}\) exchanger to SR Ca\(^{2+}\)-ATPase mRNA is altered in failing human hearts. This would be consistent with the notion that differences in the regulation of these genes emerge during the development of heart failure. The biological significance of this altered coordination remains to be determined. It should be noted, however, that in view of the decreased density of SR Ca\(^{2+}\)-ATPase pumps in the failing heart, a new equilibrium must be established together with new relations between Ca\(^{2+}\) fluxes across the sarcolemma. In this context, it is worthwhile to note the observations of Arai et al.\(^4\),\(^1\) who studied the mRNA level of several SR Ca\(^{2+}\)-ATPase regulatory proteins in the failing human heart. These investigators reported a close positive relation between the mRNA levels of the Ca\(^{2+}\)-release channel, SR Ca\(^{2+}\)-ATPase, and phospholamban, again suggesting that proteins critically involved in Ca\(^{2+}\) fluxes across the SR membrane or sarcolemma are coordinately regulated in their gene expression.

A substantial variation in the number of SR Ca\(^{2+}\)-ATPase and Na\(^+\)-Ca\(^{2+}\) exchanger transcripts was observed in both failing and nonfailing myocardium, similar to the wide range of ventricular gene expression reported recently for ANF or SR Ca\(^{2+}\)-ATPase.\(^7\)\(^,\)\(^3\)\(^9\)\(^,\)\(^4\)\(^1\)

Because of the good reproducibility of Northern blot and slot blot analysis, it appears unlikely that this variation resulted from methodological limitations. Notably, handling of tissue samples during removal, freezing, transport, and assay was standardized in all cases, and RNA gels obtained from all samples indicated no RNA degradation. Consistent with other investigators,\(^3\)\(^9\)\(^,\)\(^4\)\(^2\)\(^,\)\(^4\)\(^3\) the ANF gene expression was upregulated only in failing but not in nonfailing human hearts. These findings are consistent with the patients’ clinical characteristics, supporting the presence of advanced heart failure. Indeed, ANF is considered to represent a well-defined marker gene of cardiac hypertrophy whose gene expression is upregulated in advanced chronic heart failure.\(^5\)\(^,\)\(^2\)\(^4\) Conversely, the very low expression of ANF in our nonfailing tissues supports the notion that the samples of these “donors” were indeed normal. Finally, the favorable correlations of SR Ca\(^{2+}\)-ATPase mRNA to cardiac index and ejection fraction may suggest that the severity of left ventricular dysfunction is an important determinant of SR Ca\(^{2+}\)-ATPase gene expression.

In tissues consisting of various cell types, such as the heart, different amounts of myocardial cells might be present, depending on the extent of fibrosis versus hypertrophy. Hence, the quantification of myocyte-specific gene expression in relation to parameters not specific for cardiac myocytes (such as 18S rRNA) may be influenced by alterations in nonmyocyte cell proliferation. Therefore, quantification of Na\(^+\)-Ca\(^{2+}\) exchanger and SR Ca\(^{2+}\)-ATPase mRNA levels obtained by slot blot analysis was expressed in relation to 18S rRNA and myocyte-specific β-MHC mRNA, respectively. Both parameters yielded, in principle, similar results, although statistical significance was missed in some of the intergroup comparisons. The mRNA levels of both Na\(^+\)-Ca\(^{2+}\) exchanger and SR Ca\(^{2+}\)-ATPase were translated into significant corresponding changes of protein levels, suggesting that the transition into SR Ca\(^{2+}\)-ATPase and Na\(^+\)-Ca\(^{2+}\) exchanger protein is regulated at the pretranslational level. The yields of both myocyte-specific β-MHC protein and protein of the particulate fraction used for normalization of SR Ca\(^{2+}\)-ATPase and Na\(^+\)-Ca\(^{2+}\) exchanger protein levels were comparable and not significantly different in nonfailing and failing human hearts. Thus, we believe that the present data demonstrate that the expression of the Na\(^+\)-Ca\(^{2+}\) exchanger is increased in the failing human heart because of both coronary artery disease and dilated cardiomyopathy, whereas the expression of the SR Ca\(^{2+}\)-ATPase is decreased. Interestingly, although the gene expression of Na\(^+\)-Ca\(^{2+}\) exchanger and SR Ca\(^{2+}\)-ATPase did not differ significantly between patients with coronary artery disease and dilated cardiomyopathy, the ratio of Na\(^+\)-Ca\(^{2+}\) exchanger mRNA to SR Ca\(^{2+}\)-ATPase mRNA was substantially higher in patients with dilated cardiomyopathic myocardium. This may indicate that a higher compensatory activity of the Na\(^+\)-Ca\(^{2+}\) exchanger is required in the latter. In this context, Brillantes et al.\(^4\) have recently reported that the expression of the ryanodine receptor is decreased selectively in ischemic cardiomyopathy, indicating distinct differences in intracellular Ca\(^{2+}\) handling in dilated cardiomyopathy versus coronary artery disease.

What are the potential functional implications of an increased Na\(^+\)-Ca\(^{2+}\) exchanger gene expression and enhanced abundance of protein levels in the face of decreased SR Ca\(^{2+}\)-ATPase pumps? The reduced rate of the Ca\(^{2+}\) uptake by the SR is considered to be the cause for the prolonged cytosolic Ca\(^{2+}\) transients with retarded diastolic Ca\(^{2+}\) decline, as measured in overloaded or failing myocardium in vitro by using Ca\(^{2+}\)-sensitive indicators.\(^5\)\(^,\)\(^6\)\(^,\)\(^4\)\(^6\). Indeed, several studies demonstrated a reduced in vitro Ca\(^{2+}\) accumulation into SR vesicles isolated from human dilated cardiomyopathy or from animal models with overload hypertrophy.\(^5\)\(^,\)\(^6\)\(^,\)\(^4\)\(^6\) However, Movsesian and colleagues,\(^5\)\(^,\)\(^4\)\(^7\) investigating highly purified SR vesicles of human failing heart, did not observe reduced SR Ca\(^{2+}\) uptake. It has been speculated that the discrepancy between these studies is due to methodological differences, such as the utilization of whole-muscle homogenate versus purified SR vesicles for the Ca\(^{2+}\) uptake studies.\(^4\)\(^8\) Similarly, functional data of Na\(^+\)-Ca\(^{2+}\) exchange obtained by analyzing kinetics of sodium accumulation in membrane vesicles are subject to methodological criteria\(^2\) and demonstrate contradictory results in animal models of cardiac over-
load;17-20; thus, quantitative analysis of Na⁺-Ca²⁺ exchange remains elusive unless the intracellular Na⁺ concentrations under normal and pathological conditions are known.49

It is reasonable to assume that the increased abundance of Na⁺-Ca²⁺ exchanger mRNA and protein reflects increased functional activity, i.e., an increased number of Na⁺-Ca²⁺ exchanger molecules. If so, then enhanced expression of sarcotemmal Na⁺-Ca²⁺ exchanger might be considered as a compensatory adaptation to the failure-associated SR dysfunction, since the Na⁺-Ca²⁺ exchanger contributes to the diastolic Ca²⁺ removal from the cytosol by using the sodium gradient as a driving force.2,50 However, this compensation might involve an arrhythmogenic component: Ca²⁺ removal by the exchanger is electrogenic, causing depolarization due to the coupling of one Ca²⁺ to the influx of three Na⁺. In cases of acute substantial cytosolic Ca²⁺ overload, the enhanced activation of the Na⁺-Ca²⁺ exchanger appears to contribute to the occurrence of delayed afterdepolarizations and triggered activity.51 Vice versa, given the putative significance of Na⁺-Ca²⁺ exchange in cardiac excitation-contraction coupling,15,16 increased Ca²⁺ influx by the Na⁺-Ca²⁺ exchanger during the action potential may be associated with a positive inotropic effect, which, again, may reflect a compensatory mechanism in the failing heart.

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