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Sarcoplasmic reticulum Ca^{2+}ATPase and phospholamban mRNA and protein levels in end-stage heart failure due to ischemic or dilated cardiomyopathy

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Abstract Abnormalities in intracellular Ca^{2+} handling play a crucial role in the pathogenesis of heart failure. The reduced capacity of failing human myocardium to restore low resting Ca^{2+} levels during diastole has been explained by the impairment of Ca^{2+} uptake into the sarcoplasmic reticulum (SR) via the SR Ca^{2+}ATPase. It is unclear whether Ca^{2+}ATPase function, protein levels, and mRNA steady-state levels correspond to one other, and whether the cause of heart failure, namely idiopathic dilated or ischemic cardiomyopathy, produces different changes. The present study examined SR Ca^{2+}ATPase activity and both mRNA and protein levels of SR Ca^{2+}ATPase, phospholamban, and $Gi\alpha_2$ in left ventricular myocardium from eight nonfailing hearts, from eight hearts of patients with idiopathic dilated cardiomyopathy (DCM), and from six hearts from patients with ischemic cardiomyopathy (ICM). Compared to nonfailing myocardium, the activity of the SR Ca^{2+}ATPase was significantly reduced in failing myocardium from patients with DCM (36%, $P<0.01$) and from patients with ICM (37%, $P<0.001$). Significantly lower levels of SR Ca^{2+}ATPase mRNA levels (55% and –56%, $P<0.001$ for DCM and ICM, respectively) and phospholamban mRNA (45%, $P<0.001$ for DCM; 31%, $P<0.05$ for ICM) were observed in failing than in nonfailing myocardium. In contrast, no significant changes were observed at the level of proteins. $Gi\alpha_2$ mRNA and protein levels were both significantly increased in failing mycocardium. There were no differences between idiopathic dilated and ischemic cardiomyopathy concerning the examined parameter. It is concluded that reduced SR Ca^{2+}ATPase activity contributes to an altered intracellular Ca^{2+} handling by the SR in both dilated and ischemic cardiomyopathic hearts. However, changes in SR Ca^{2+}ATPase and phospholamban steady-state protein levels do not contribute to these alterations. The dissociation between protein and mRNA levels provides evidence for a posttranscriptional or posttranslational regulation of these proteins. The observed alterations are not dependent on the underlying cause of end-stage heart failure.

Key words Heart failure · Human left ventricle · Sarcoplasmic reticulum Ca^{2+}ATPase · Phospholamban · cAMP

Abbreviations *GAPDH* Glyceraldehyde-3-phosphate dehydrogenase · *$Gi\alpha_2$* Inhibitory G-protein α-subunit · *β-MHC* β-Myosin heavy chain · *PAGE* Polyacrylamide gel electrophoresis · *SDS* Sodium dodecylsulphate · *SR* Sarcoplasmic reticulum

Introduction

Abnormalities in intracellular Ca^{2+} handling as a cause for both systolic and diastolic dysfunction are thought to play a crucial role in the pathogenesis of heart failure in dilated cardiomyopathy [1]. In the failing human myocardium the time course of contraction, rate of tension rise, and especially time of relaxation are prolonged under basal [2] and stimulated conditions [3]. This can be explained in part by a reduced capacity of the failing heart to restore low resting Ca^{2+} levels during diastole [2, 4–7]. Disturbances in diastolic Ca^{2+} homeostasis seem to be due primarily to impaired uptake of Ca^{2+} into the sarcoplasmic reticulum (SR), as has been shown in several different animal models [8–11] and in human dilated cardiomyopathy [7, 12–14]. In contrast to these findings, Movsesian et al. [15], performing experiments on enriched fractions of isolated SR vesicles from human hearts, observed no alterations in Ca^{2+} uptake by the SR in failing compared to nonfailing human hearts.

Abnormalities in diastolic SR Ca^{2+} handling may be explained by three possible mechanisms. The first is a reduction in the amount of SR per cell. The second pos-

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sible cause of an altered Ca^{2+} homeostasis are alterations in the β-adrenoceptor adenylate cyclase system. Downregulation of β-adrenoreceptors [16], increase in inhibitory guanine nucleotide-binding proteins [17–19], and decrease in cAMP formation [20] may lead to a decrease in cAMP-dependent protein kinase activity in the intact cell and thereby to reduced phosphorylation of phospholamban. Impaired phosphorylation of phospholamban would result in deficient activation of the SR Ca^{2+}ATPase because of a persistent inhibition by the nonphosphorylated form of phospholamban, in turn leading to decreased diastolic uptake of Ca^{2+} into the SR [21–25]. Finally, a decrease in the levels of SR proteins themselves with a consequently reduced SR Ca^{2+}ATPase activity could produce adverse effects on diastolic Ca^{2+} sequestration in heart failure. Steady-state levels of mRNA for SR Ca^{2+}ATPase and phospholamban have been shown to be reduced in many different animal models of cardiac overload or heart failure [9–11, 26] and in human dilated cardiomyopathy [13, 27–31]. Nevertheless, while in some animal models of cardiac hypertrophy the reduction in SR Ca^{2+}ATPase mRNA is accompanied by a decrease in the level of proteins [10, 32], recent data concerning SR Ca^{2+}ATPase and phospholamban protein levels in human dilated cardiomyopathy have been contradictory [13, 29, 33].

In this study SR Ca^{2+}ATPase activity was determined in nonfailing and in failing hearts of patients with either idiopathic dilated cardiomyopathy or with ischemic cardiomyopathy due to coronary artery disease. In left ventricular myocardium of the same hearts SR Ca^{2+}ATPase and phospholamban mRNA and proteins levels were also examined. The protein and mRNA levels of the inhibitory G protein α-subunit ($Gi\alpha_2$) were studied to determine whether the studied hearts reveal typical alterations described before for the failing myocardium [19, 34]. Special emphasis was put on possible differences between ischemic and dilated cardiomyopathy since differences between these conditions leading to end-stage heart failure have been reported for changes in the β-adrenoreceptor adenylate cyclase system [35] and for changes in cardiac calcium release channel expression [36], indicating distinct molecular defects underlying these two types of heart muscle diseases.

Methods and materials

Human myocardial tissue

Myocardial tissue was obtained during cardiac transplantation. Failing myocardium was obtained from eight patients with idiopathic dilated cardiomyopathy and from six patients with heart failure due to severe ischemic heart disease (ischemic cardiomyopathy). The hemodynamic and biographical data are given in the Table 1. Medical therapy consisted of cardiac glycosides, diuretics, and nitrates and the angiotensin-converting enzyme inhibitor enalapril. Patients who received catecholamines or β-adrenoceptor antagonists were withdrawn from the study. All patients gave written informed consent prior to operation. Myocardial tissue from eight nonfailing hearts that could not be transplanted was studied for comparison (4 male, 4 female, age 50.9±1.99 years). Patient history and two-dimensional echocardography revealed no evidence for heart disease. Cardiac catheterization was not performed in patients without heart failure. These patients had been ventilated for 48 h before tissue procurement. General anaesthesia was performed with flunitrazepam, fentanyl, and pancuronium bromide with isoflurane. Cardiac surgery was performed while on cardiopulmonary bypass. The cardioplegic solution used was a modified Bretschneider solution containing (in mmol/l): NaCl 15, KCl 10, $MgCl_2$ 4, histidine HCl 180, tryptophane 2, mannitol 30, and potassium dihydrogen oxoglutarate. Only noninfarcted tissue was used, and scars were carefully trimmed away. Tissue pieces were suspended in ice cold cardioplegic solution and were delivered from the operation room to the laboratory within 10–15 min and frozen immediately in liquid nitrogen.

Myocardial protein preparation

Myocardial tissue (3–5 g) was thawed on ice and chilled in 10 ml ice cold homogenization buffer (Tris/HCl 20 mmol/l, ethylenediaminetetraacetic acid 1 mmol/l, dithiotreitol 1 mmol/l, leupeptine 0.1 μmol/l, phenylmethylsulfonylfluorid 0.3 mmol/l; pH 7.4). Connective tissue was trimmed away, and the remaining tissue was minced with scissors and homogenized by hand for 1 min with a glass-glass homogenizer. The suspension was centrifuged at 100000 *g* for 30 min. The supernatant was discarded, and the pel-

Table 1 Biographical and haemodynamic data of patients with heart failure (*DCM* Idiopathic dilated cardiomyopathy, *ICM* ischemic cardiomyopathy, *NYHA* New York Heart Association grade)

Sex	Diagnosis	NYHA	Age (years)	Cardiac output (l/min)	Ejection fraction (%)	Cardiac index (min^{-1} m^{-2})
m	DCM	IV	49	3.5	25	2.1
m	DCM	IV	52	4.4	25	2.7
f	DCM	IV	51	4.3	15	2.5
f	DCM	IV	57	4.8	13	3.0
f	DCM	IV	63	4.6	20	2.8
m	DCM	IV	54	3.8	40	1.9
m	DCM	IV	52	4.7	22	2.7
f	DCM	IV	43	4.8	19	2.7
	mean±SEM		52.6±2.06	4.3 ±0.17	22.3±2.94	2.55±0.14
m	ICM	IV	63	6.4	16	3.5
m	ICM	IV	56	5.6	28	3.0
f	ICM	IV	58	4.8	38	2.9
m	ICM	IV	52	4.6	17	3.3
m	ICM	IV	59	4.3	16	2.3
f	ICM	IV	52	3.2	17	2.1
	mean±SEM		56.7±1.74	4.8 ±0.45	22±3.7	2.85±0.26

let was resuspended in 10 ml homogenization buffer and recentrifuged at 100|000 *g* for 30 min. Finally, the remaining pellet was dissolved in 5 ml of the same buffer.

Measurement of Ca^{2+}ATPase activity

The reaction was carried out according to Chu et al. [37] based on coupled enzyme reactions:

– ATP→ADP + P_i. This reaction was catalyzed by the SR Ca^{2+}ATPase.
– ADP + phosphoenolpyruvate→ATP + pyruvate. The reaction was catalyzed by the pyruvate kinase.
– Pyruvate + NADH→lactate + NAD^+. This reaction was catalyzed by the lactate dehydrogenase.

The oxidation of NADH was continuously monitored, measuring the decreasing absorbence at 340 nm using a spectrophotometer (Beckman DU 640, Beckman, Munich, Germany). The reaction was carried out in a volume of 1 ml at 37°C. Crude membranes (final concentration 50 µg protein/ml) were suspended in the reaction mixture of the following composition (in mmol/l): 3-[*N*-morpholino]propanesulfonic acid 21; sodium azide 4.9; ethylene glycol-bis(β-aminoethyl ether) 0.06; potassium chloride 100, magnesium chloride 3; phosphoenolpyruvate 1, nicotinamide hypoxanthine dinucleotide 0.2; calcium ionophore A23187 (calcimycin) 0.003, pyruvate kinase/lactate dehydrogenase mixture 8.4/12 U. Calcium chloride was added to the reaction mixture to yield desired free Ca^{2+}-concentrations calculated according to Fabiato [38]. The reaction was started with ATP (1 mmol/l) and was linear with time over at least 5 min. The basal activity was measured in the absence of Ca^{2+} and presence of ethylene glycol-bis(β-aminoethyl ether (4 mmol/l) simultaneously. All experments were carried out in triplicate. The activity of the Ca^{2+}ATPase is given in nanomoles of ATP per milligram of protein per minute. In addition, the effect of the specific Ca^{2+}ATPase inhibitor cyclopiazonic acid (and thapsigargin were also studied.

Isolation of total RNA

Total RNA from frozen left ventricular tissue samples were prepared according to the protocol of Chomczinkski and Sacchi [39]. Between 50 and 100 µg total RNA was obtained from 150 mg tissue. The amount of RNA was determined by UV absorption. The ratio of optical density 260 to 280 nm was 1.8–2.0 in all cases.

Northern blot analysis

Total RNA (10 µg) was separated in a 6% formaldehyde/1.2% agarose gel. Signs of RNA degradation were excluded by inspection of the ethidium bromide stained gel in UV light. The RNA was blotted on nylon membranes (Schleicher und Schuell, Dassel, Germany) by overnight capillary blotting and fixed by UV irradiation. After fixation the blots were prehybridized in solution containg 5x SSC, 5×Denhardt's solution, 50% formamide, 1% sodium dodecylsulphate (SDS), 50 mmol/l sodium phosphate pH 6.8, 10% dextran sulphate, 100 g/ml salmon sperma DNA at 42°C. Hybridization was performed in 50% formamide solution at 42°C for 16 h. The membrane was successively hybridized with a 2-kb cDNA fragment (*Bam*HI-*Bam*HI) encoding for rabbit SR Ca^{2+}ATPase (provided by D. H. MacLennan, Toronto) [40] and a 0.3-kb cDNA fragment (*Hind*3-*Not*1) encoding for human phospholamban (kindly provided by B. Linck, Münster) [29], which was generated by polymerase chain reaction amplification with specific primers chosen from the human phospholamban sequence published by Fujii et al [41]. The cDNA for human $Gi\alpha_2$ (1.7-kb insert, *Eco*R1-*Kpn*1) was kindly donated by J. Didsbury, Durham [42]. The fragments were excised from the plasmid vector with the appropriate restriction enzymes, separated from the vector DNA on a 1% low-melt agarose gel, and labeled with [α-^{32}P]dCTP (Amersham Buchler, Braunschweig, Germany) using the Multiprime DNA Labeling Kit from Amersham Buchler (Braunschweig, Germany). The concentration of the labeled probe in the hybridization solution was 1×10^6 cpm/ml.

After hybridization with the SR Ca^{2+}ATPase, phospholamban, or $Gi\alpha_2$ cDNA fragments the membrane was successively washed twice in 2x SSC/0.1% SDS at room temperature at 42° C for 15 min and twice in 0.2x SSC/0.1% SDS at 68°C for 45 min. Standardization was performed by hybridization of the same membrane using a 40-base single stranded synthetic oligonucleotide probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Dianova, Hamburg, Germany). Hybridization conditions were the same as described above. Stringency washes were performed with 2x SSC/0.1% SDS at room temperature, for 30 min with 2x SSC/0.1% SDS at 65° C, and twice for 5 min with 2x SSC/0.1% SDS at room temperature. Membranes were exposed to Kodak films (Kodak X-OMAT, Kodak, Rochester, USA). Exposure time was between 36 h (SR Ca^{2+}ATPase) and 5–7 days (phospholamban). Different exposure times – especially for GAPDH signals – did not significantly alter the results of quantification. The mRNA size of the detected signals was estimated by relating their position to the position of the 18S and 28S ribosomal RNA as detected in the ethidium bromide stained electrophoresis gel in UV light. Quantification of the signals was performed by denstometric analysis using the Image Quant Densitometric System (Molecular Dynam, Krefeld, Germany), which allowed wide-line integration to determine average density values within reactangles surrounding the hybridization signals. For each blot the quantification procedure was repeated three times in order to confirm the results. Relative amounts of specific RNA were calculated by relating the densitometric values of the single signals to the densitometric values of the hybridzation signal for GAPDH, which was used as a standard for the amount of RNA loaded on the gel. For standardization between different northern blots, a human myocardial reference RNA was used, which was loaded on each gel.

Immunoblotting techniques

Immunoblotting techniques were performed as previously described, with slight modifications [19, 43]. Samples of 50 µg myocardial proteins were denatured by heating to 95°C in electrophoresis buffer containing 4% SDS, 50 mmol/l Tris/HCl pH 6.8, 20% glycerol, 0.005% pyronin, and 10 mmol/l dithiotreitol. Separation of proteins was performed by SDS-polyacrylamide gel electrophoresis [SDS-PAGE, 10% (wt/vol.) acrylamide/for β-myosin heavy chain (β-MHC) 7.5% (wt/vol.), 16 cm length]. After electrophoretic separation proteins were transferred to a nitrocellulose membrane (0.2 µm; Schleicher und Schuell, Dassel, Germany) by semidry electrophoretic blotting using a LKB 2117-250 Novablot Electrophretic Transfer Kit (LKB-Pharmacia, Freiburg, Germany; 0.8 mA/cm^2, 90 min) with a discontinuous buffer system according to the method of Towbin et al [44]. Electrophoretic transfer was controlled by staining the nitrocellulose membranes with Ponceau red. The sheets were immersed in 100 ml 5% low fat dry milk powder in phosphate-buffered saline buffer (KH_2PO_4 100 mmol/l; NaCl 137 mmol/l; KCl 2.68 mmol/l, $NaH_2PO_4\times H_20$ 10.44 mmol/l; pH 7.4) and shaken for at least 1 h at room temperature. After repeated washes in phosphate-buffered saline/Tween-20 (0.5% v/v) the sheets were incubated with the first antibody. Phospholamban was detected using a phospholamban monoclonal antibody (A1, diluted 1:500) raised against purified phospholamban from canine hearts, which was purchased from Dianova (Hamburg, Germany), SR Ca^{2+}ATPase using a monoclonal specific antibody raised in mouse (dilution 1:2500), which was purchased from Dianova (Hamburg, Germany). For determination of Giα a polyclonal antiserum (MB1, diluted 1:2000) was used which had been raised in rabbits against the C-terminal decapeptide of retinal transducin (KENLKDCCGLF) [19]. The antiserum recognized $Gi\alpha_1$ and $Gi\alpha_2$ as studied previously using recombinant G protein subunits [19]. Since in the human heart only $Gi\alpha_2$ and $Gi\alpha_3$ are present [19], in human myocardial preparations the MB1 immunoreactive material detected in these myocardial sam-

ples corresponds to $Gi\alpha_2$ only. For detection of β-MHC a monoclonal specific antibody raised against bovine β-MHC in mouse (diluted 1:500) was purchased from Biocytex (Marseille, France). The antibody was specific for β-MHC (J.J. Leger, Montpellier, personal communication). After repeated washes immunoreaction was continued by incubation of the nitrocellulose sheets with a peroxidase-conjugated goat secondary antibody raised against rabbit, mouse, and canine IgG, respectively. Detection was performed using the enhanced chemoluminiscence assay (ECL-Kit, Amersham, Braunschweig, Germany). After exposure to X-ray film (Kodak X-OMAT AR, Kodak, Rochester, USA), signals were quantified by two dimensional densitometry (Image Quant Densitometric System, Molecular Dynamics, Krefeld, Germany) as described earlier. Before quantification of immunoreactive phospholamban and SR Ca^{2+}ATPase it had been carefully ensured that immunodetection of the separated proteins on western blots was in the linear range, and that electrophoretic transfer was complete in the relevant molecular weight range. There was no loss of protein due to overblotting as studied with Ponceau red stains of nitrocellulose sheets.

Miscellaneous

Protein concentrations were determined according to Lowry et al. [45] using bovine serum albumin as standard. 5′-Nucleotidase activity was analyzed with the method of Dixon and Purdom [46].

Materials

Antibodies, unless otherwise indicated, were obtained from Sigma-Aldrich (Deisenhofen, Germany). Restriction enzymes were purchased from Boehringer (Mannheim, Germany). All other chemicals were of analytical grade or the best grade commercially available. Only deionized and double-destilled water was used throughout.

Statistics

Data shown are mean+SEM. Statistical significance was estimated with Student's *t* test for unpaired observations and analysis of variance according to Wallenstein et al. [47]. A value of $P<0.05$ was considered significant.

Results

Ca^{2+}ATPase measurement in human sarcoplasmic reticulum

SR Ca^{2+}ATPase was measured in the presence of the calcium ionophore A23187 (3 μmol/l) in preparations of left ventricular myocardium from healthy organ donors and from patients with dilated cardiomyopathy or ischemic cardiomyopathy. Increasing concentrations of free Ca^{2+} (up to 300 μmol/l) led to a concentration-dependent increase in the activity of the SR Ca^{2+}ATPase in all groups. Mean values of maximal SR Ca^{2+}ATPase activity (free Ca^{2+} concentration 79.4 μmol/l) are shown in Fig. 1. In nonfailing myocardium the maximal Ca^{2+}ATPase activity was 243±21 nmol ATP mg^{-1} protein min^{-1}, in failing myocardium due to dilated cardiomyopathy 155.6±22 nmol ATP mg^{-1} protein min^{-1} and in failing myocardium due to ischemic cardiomyopathy 153.4±18 nmol ATP mg protein min^{-1}. Thus, in failing myocardium Ca^{2+}ATPase activity was reduced by 36%

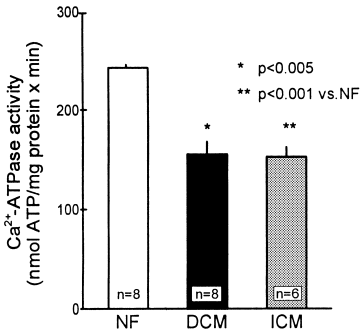

Fig. 1 Maximal SR Ca^{2+}ATPase activity in crude membrane preparations of left ventricular myocardium from nonfailing (*NF*) and failing myocardium from patients with idiopathic dilated cardiomyopathy (*DCM*) and ischemic heart disease (*ICM*). SR Ca^{2+}ATPase activity was determined in the presence of the ionophor A23187 (3 μmol/l) and increasing concentrations of free Ca^{2+} (up to 300 μmol/l). SR Ca^{2+}ATPase activity was increased by free Ca^{2+} in a dose-dependent manner with the maximum at 79.4 μmol/l

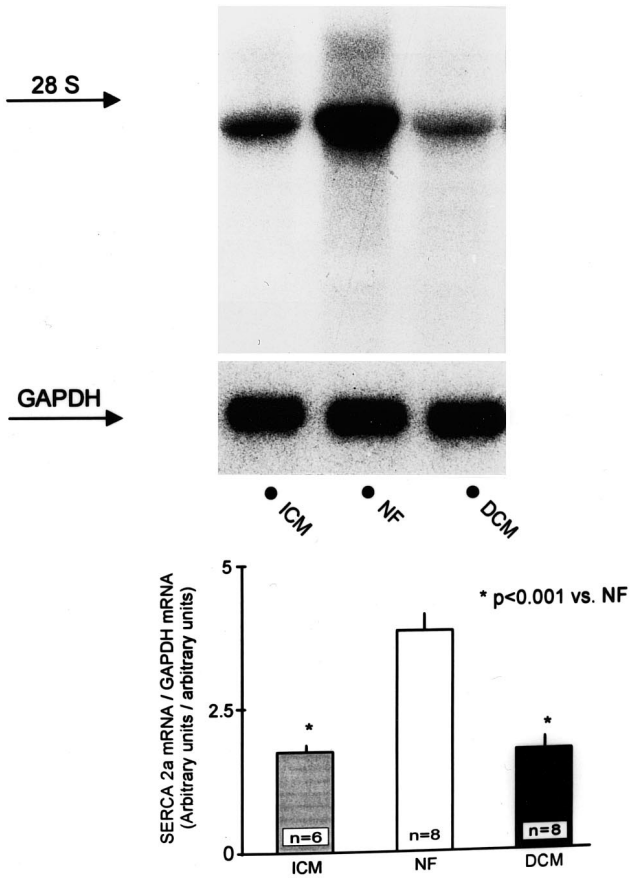

Fig. 2 Result of northern blot analysis of total RNA from nonfailing left ventricular mycardium (*NF*) and left ventricular myocardium from patients with idiopathic dilated cardiomyopathy (*DCM*) and ischemic cardiomyopathy (*ICM*). On each lane 10 μg total RNA was loaded. *Above*, representative hybridization signals for SR Ca^{2+}ATPase (*SERCA2a*), which occurred at a position just below the position of the 28S RNA corresponding to a mRNA size of approximately 4 kb. Signal for GAPDH mRNA is shown as control. *Below*, mean values (±SEM) of relative SR Ca^{2+}ATPase mRNA levels related to GAPDH mRNA levels in left ventricles from NF, DCM, and ICM

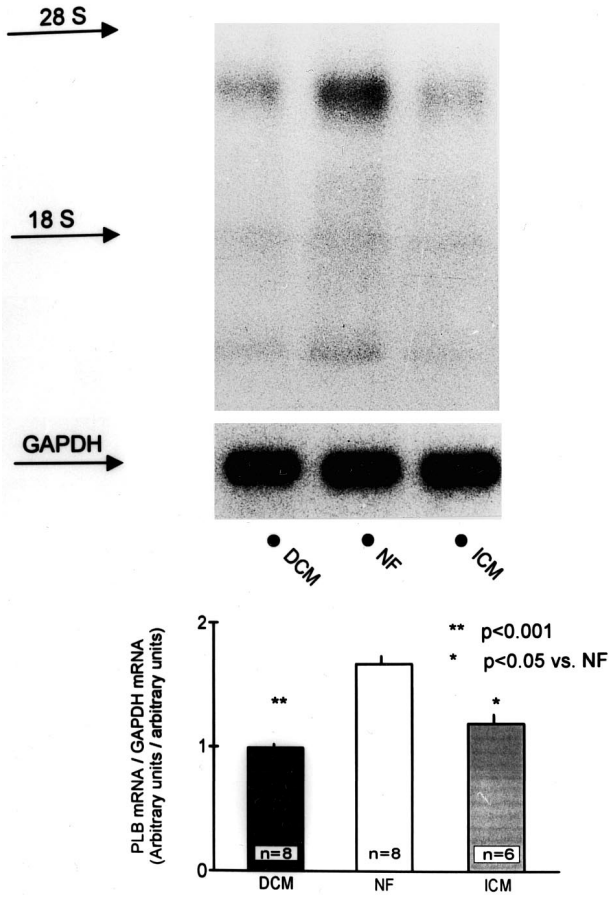

Fig. 3 Result of northern blot analysis of total RNA from nonfailing left ventricular mycardium (*NF*) and left ventricular mycardium from patients with idiopathic dilated cardiomyopathy (*DCM*) and ischemic cardiomyopathy (*ICM*). *Above*, representative hybridization signals for phospholamban mRNA (*PLB*). Three distinct bands were obtained corresponding to a mRNA size of 3.3, 1.9, and 0.6 kb. The strongest signal at 3.3 kb corresponds to the size of PLB. Hybridization signal for GAPDH is shown as control. *Below*, mean values (±SEM) of relative PLB mRNA levels related to GAPDH mRNA levels in left ventricles from NF, DCM, and ICM

compared to nonfailing myocardium (P<0.01 for dilated cardiomyopathy and P<0.001 for ischemic cardiomyopathy), regardless of the underlying cause of heart failure. The presence of the Ca^{2+}ATPase inhibitors cyclopiazonic acid and thapsigargin depressed Ca^{2+}ATPase activity in a concentration-dependent manner (data not shown).

Relative levels of sarcoplasmic reticulum Ca^{2+}ATPase and phospholamban mRNA

Figure 2 shows hybridization signals and mean values for SR Ca^{2+}ATPase mRNA. A single signal for SR Ca^{2+}ATPase occurred at a position just below the position of the 28S RNA, corresponding to a mRNA size of approximately 4 kb (Fig. 2, upper panel). Hybridization with the 300-bp cDNA fragment encoding phospholamban revealed three distinct bands corresponding to a mRNA size of 3.3, 1.9, and 0.6 kb (Fig. 3, upper panel). The strongest signal was obtained at 3.3 kb, which was in accordance with the original findings by Fujii et al. [41] who explained the other bands as poly(A) attachement sites or transcriptional initiation sites. As depicted in Fig. 2, lower panel, mean values for relative SR Ca^{2+}ATPase mRNA levels relative to GAPDH mRNA levels were decreased by 55% and 56% in myocardium from patients with dilated cardiomyopathy (P<0.001) and ischemic cardiomyopathy (P<0.001) respectively compared to nonfailing myocardium. Similarily, relative phospholamban mRNA levels were reduced by 45% in left ventricular myocardium from patients with dilated cardiomyopathy (P<0.001) and by 31% in myocardium from patients with ischemic cardiomyopathy (P<0.05; Fig. 3, lower panel). No significant differences were observed between idiopathic dilated and ischemic cardiomyopathy (Table 2).

Table 2 mRNA steady state levels of SR Ca^{2+}ATPase, phospholamban, and guanine-nucleotide binding inhibitory protein $Gi\alpha_2$ nonfailing and failing human left ventricular myocardium (for standardization, data are given relative to GAPDH mRNA levels)

	non failing (n=8)	idiopathic dilated cardiomyopathy (n=8)	ischemic heart disease (n=6)
SRCA2a	3.75 ±0.25	1.69 ±0.33**	1.76 ±0.11**
phospholamban	1.72 ±0.09	0.95 ±0.06**	1.18 ±0.11*
$Gi\alpha_2$	0.22 ±0.02	0.30 ±0.03*	0.29 ±0.02*

* P<0.05, ** P<0.001

Protein levels of sarcoplasmic reticulum Ca^{2+}ATPase and phospholamban

For SR Ca^{2+}ATPase and phospholamban no significant differences between failing and nonfailing myocardium were detected at the level of proteins. Figures 4 and 5 show representative results of western blot analysis of SR Ca^{2+}ATPase and phospholamban using highly specific monoclonal antibodies. Figure 6 shows a representative western blot indicating – exemplary for SR Ca^{2+}ATPase protein – that immunochemical detection was within the linear range. Immunochemical detection of SR Ca^{2+}ATPase revealed a single band at the expected position of approx. 100 kDa (Fig. 4, upper panel). In some cases a second weak band occurred immediately underneath the main signal, which could represent either a weak cross-reactivity of the antibody with another protein or a degradation product. Immunochemical detection of phospholamban in unboiled protein samples revealed a single band at the expected position of 29 kDa (Fig. 5, upper panel). As can be seen on the lower panels of Figs. 4 and 5, no difference was detected between failing and nonfailing myocardium concerning mean SR Ca^{2+}ATPase or phospholamban protein levels. For pho-

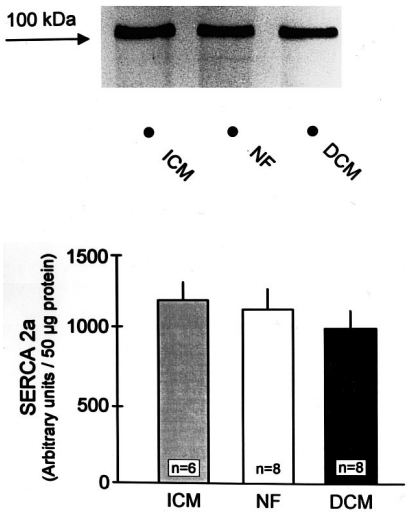

Fig. 4 Result of the immunochemical detection (western blots) of SR Ca^{2+}ATPase (*SERCA2a*, M_r ≈100 kDa). Raw homogenates were prepared from nonfailing left ventricular myocardium (*NF*) and from left ventricular myocardium from patients with ischemic cardiomyopathy (*ICM*) and with idiopathic dilated cardiomyopathy (*DCM*). Separation of 50 µg protein per lane was performed by 10% SDS-PAGE before electrophoretic transfer to nitrocellulose membranes. *Above*, representative signals. *Below*, mean values (±SEM) from densitometric analysis. Note that there are no significant differences between the three groups. Results were not significantly altered when SERCA2a protein values were related to 5′-nucleotidase activity and to β-MHC protein levels

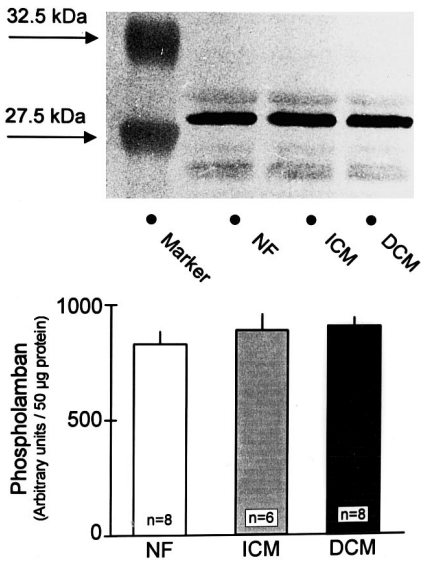

Fig. 5 Result of the immunochemical detection (western blots) of phospholamban (PLB, M_r ≈29 kDa). Raw homogenates were prepared from nonfailing left ventricular myocardium (*NF*) and from left ventricular myocardium from patients with ischemic cardiomyopathy (*ICM*) and with idiopathic dilated cardiomyopathy (*DCM*). Separation of 50 µg protein per lane was performed by 10% SDS-PAGE before electrophoretic transfer to nitrocellulose membranes. *Above*, representative signals. *Outer left lane*, Poinceau red stained molecular weight marker. *Below*, mean values (±SEM) from densitometric analysis. Note that there are no significant differences between the three groups. Results were not significantly altered when PLB protein values were related to 5′-nucleotidase activity and to β-MHC protein levels

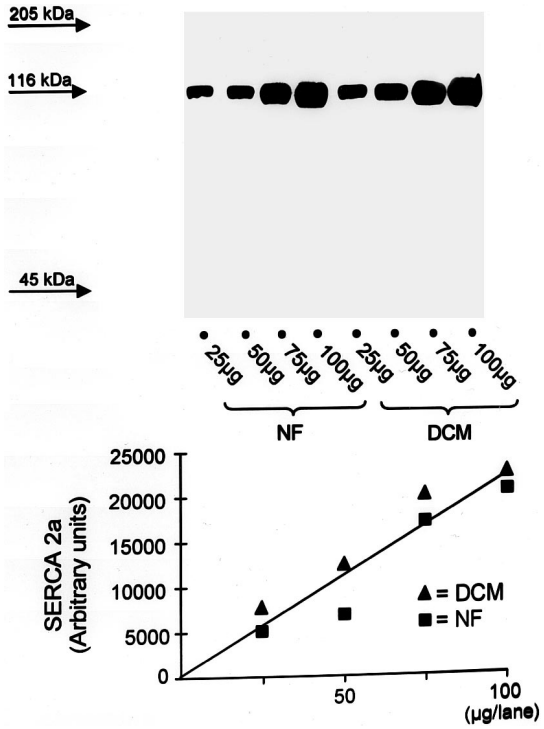

Fig. 6 Result of the immunochemical detection (western blot) of SR Ca^{2+}ATPase (*SERCA2a*, M_r ≈100 kDa). Same method as described in Fig. 4. Myocardial protein (10, 25, 50, and 100 µg) of from a nonfailing (*NF*) and failing (*DCM*) human left ventricle was separated on 10% SDS-PAGE before electrophoretic transfer to a nitrocellulose membrane. Notice that immunochemical detection was within the linear range and that the antibody reacted highly specifically with SR Ca^{2+}ATPase protein

spholamban the results were similar for the oligomeric form (approx. 29 kDa) and the monomeric (approx. 8 kDa) – obtained after boiling of the protein samples prior to electrophoresis – or the sum of the two were quantified (not shown).

For SR Ca^{2+}ATPase the results were not altered when the second weak band was also quantified. In the experiments shown, 10% polycacrylamide gels were used and loaded with 50 µg protein per lane. There was no significant difference between failing and nonfailing myocardium when the data from densitometric analysis were relative to values for β-MHC protein levels or to 5′-nucleotidase activity or to total protein. Nevertheless, as can be seen in Table 3, specific relations between mean values changed when different ratios were calculated. For example, while mean values for SR Ca^{2+}ATPase and phospholamban protein levels were slightly increased in ischemic cardiomyopathy myocardium compared to mean values in nonfailing myocardium, when densitometric units were related only to the amount of protein used on PAGE, they were decreased compared to nonfailing myocardium when densitometric units where related to β-MHC values. β-MHC values themselves showed a tendency towards higher values in failing myocardium compared to normal myocardium. None of the

observed differences reached statistical significance (Fig. 7). Absolute values for 5′-nucleotidase are given in Table 3.

mRNA and protein levels of $Gi\alpha_2$

It has been reported before that both mRNA [34] and protein levels [18, 19] of inhibitory G proteins are increased in failing human myocardium. In the present study $Gi\alpha_2$ protein and mRNA were studied to ensure that the studied samples exhibit typical changes observed in failing human myocardium. This measurement of $Gi\alpha_2$ mRNA and protein also served as a control that the techniques used here are also able to detect increases in protein and mRNA steady-state levels. Hybridization signal and mean values for $Gi\alpha_2$ mRNA are shown in Fig. 8. A single band occurred at the expected position

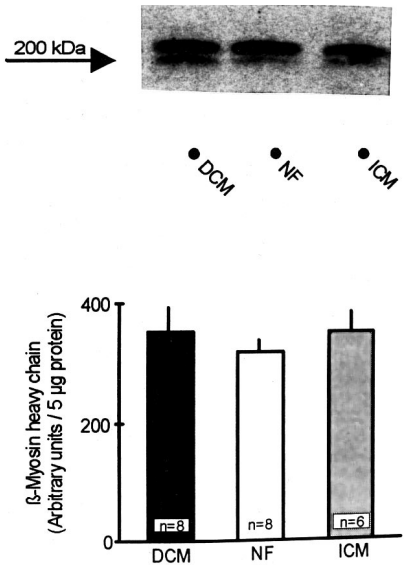

Fig. 7 Result of the immunochemical detection (western blots) of β-MHC protein. Raw homogenates were prepared from nonfailing left ventricular myocardium (*NF*) and from left ventricular myocardium from patients with ischemic cardiomyopathy (*ICM*) and with idiopathic dilated cardiomyopathy (*DCM*). Protein samples were denatured by heating to 95° C for 5 min in electrophoresis buffer containing 4% SDS, 50 mmol/l Tris/HCl pH 6.8, 20% glycerol, 0.005% pyronin, and 10 mmol/l dithiotreitol. Separation of 5 µg protein per lane was performed by 7.5% SDS-PAGE before electrophoretic transfer to nitrocellulose membranes. Above, representative signals. *Below*, mean values (±SEM) from densitometric analysis

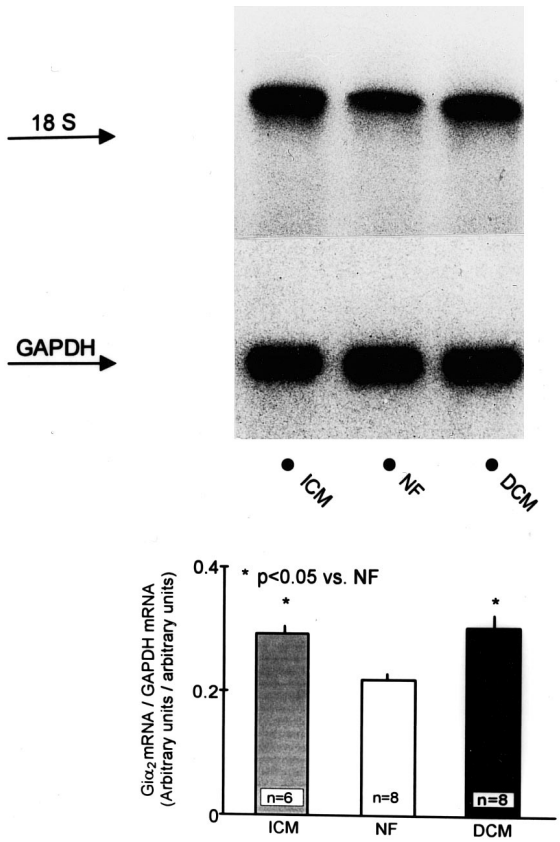

Fig. 8 Evaluation of $Gi\alpha_2$ mRNA levels from nonfailing left ventricular myocardium (*NF*) and left ventricular myocardium from patients with idiopathic dilated cardiomyopathy (*DCM*) and ischemic cardiomyopathy (*ICM*) was performed by northern blots analysis. On each lane 10 µg total RNA was loaded. *Above*, representative hybridization signals for $Gi\alpha_2$ mRNA, which occurred at a position corresponding to a mRNA size of approximately 2.4 kb. Signal for GAPDH mRNA is shown as control. *Below*, mean values (±SEM) of $Gi\alpha_2$ mRNA levels relative to GAPDH mRNA levels in left ventricles from NF, DCM, and ICM. Values were significantly decreased in failing myocardium compared to nonfailing

Table 3 Protein concentrations of SR Ca^{2+}ATPase (*SERCA2a*), phospholamban (*PLB*) and guanine nucleotide binding inhibitory protein $Gi\alpha_2$ and 5′-nucleotidase activity nonfailing and failing human left ventricular myocardium (SERCA 2a, PLB, and β-MHC given in densitometric units, 5′-nucleotidase activity in nmol P_i/mg protein in 20 min)

	non failing (*n*=8)	idiopathic dilated cardiomyopathy (*n*=8)	ischemic heart disease (*n*=6)
SERCA2a/50 µg protein	1145±146	1017±114	1206±138
SERCA2a/5′-nucleotidase	476±54	338±60	486±49
SERCA2a /β-MHC	3.6±0.52	3.1±0.3	3.5±0.39
PLB/50 µg protein	830±55	912±37	889±69
PLB/5′-nucleotidase	345±17.3	326±21.2	357±24.2
PLB/β-MHC	2.8±0.16	2.9±0.23	2.51±0.15
$Gi\alpha_2$/50 µg protein	1.03±0.098	1.84±0.12**	1.58±0.17**
$Gi\alpha_2$/5′-nucleotidase	0.42±0.03	0.7±0.09*	0.65±0.10*
$Gi\alpha_2$/β-MHC	3.22±0.25	5.39±0.37*	4.48±0.24*
5′-nucleotidase activity	2.62±0.19	2.83±0.35	2.53±0.20

* $P<0.05$, ** $P<0.001$

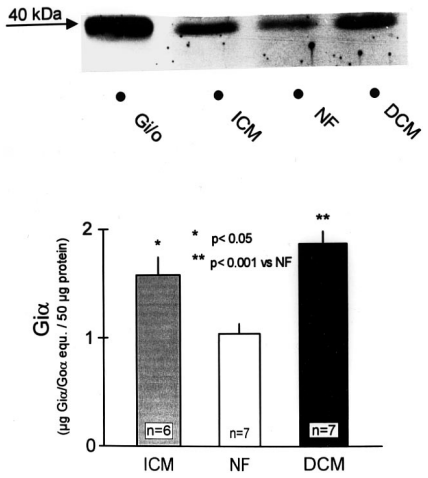

Fig. 9 Result of the immunochemical detection (western blots) of $Gi\alpha_2$ (M_r ≈40 kDa). Raw homogenates were prepared from nonfailing left ventricular myocardium (*NF*) and from left ventricular myocardium from patients with ischemic cardiomyopathy (*ICM*) and with idiopathic dilated cardiomyopathy (*DCM*). Separation of 50 µg protein per lane was performed by 10% SDS-PAGE before electrophoretic transfer to nitrocellulose membranes. *Above*, representative signals. *Below*, mean values (±SEM) from densitometric analysis

corresponding to a mRNA size of 2.4 kb. When compared to myocardium from nonfailing hearts, relative levels of $Gi\alpha_2$ mRNA related to values for GAPDH mRNA were significantly higher in left ventricular myocardium from patients with dilated cardiomyopathy ($P<0.05$) and in left ventricular myocardium from patients with heart failure due to ischemic cardiomyopathy ($P<0.05$). Figure 9 shows the representative signals obtained from quantitative immunochemical detection of Giα (M_r approx. 40 kDa). The signal is markedly increased in both kinds of failing myocardium, due to dilated cardiomyopathy and due to ischemic cardiomyopathy. As shown in Fig. 9 (lower panel) for Giα proteins, a significant increase by 79% in myocardium from patients with dilated cardiomyopathy ($P<0.001$) and by 52% ($P<0.05$) in myocardium from patients with ischemic cardiomyopathy compared to nonfailing myocardium was observed. Similar results were obtained when Giα protein levels were related β-MHC and to 5′-nucleotidase activity. As for SR Ca^{2+}ATPase and phospholamban, no significant differences were observed between relative levels of $Gi\alpha_2$ mRNA and protein levels of the two subtypes of failing myocardium themselves.

Discussion

This study demonstrates that there is a significant difference between the SR Ca^{2+}ATPase activity in left ventricular myocardium from failing and from nonfailing human hearts which is not accompanied by similar changes in left ventricular protein levels of SR Ca^{2+}ATPase and phospholamban. In failing myocardium SR Ca^{2+}ATPase activity was 36% lower than in nonfailing myocardium. These data are in accordance with those reported previously by de la Bastie et al. [9] and Levitsky et al. [10] in an animal model of pressure overload-induced cardiac hypertrophy in rats, where a decreased Ca^{2+} uptake into the SR was observed, and with those reported by Tomplison et al. [48], who demonstrated a significant reduction in SR Ca^{2+}ATPase activity in an animal model of doxyrubicin-induced cardiomyopathy in dogs. Therefore there is evidence that alteration of Ca^{2+} uptake into the SR, which is probably due to an impaired function of SR Ca^{2+}ATPase, occurs in various species when an increased workload is imposed on the heart or heart failure develops. Nevertheless, data concerning changes in SR Ca^{2+} uptake rate in failing human myocardium have proven contradictory.

Recent data on the Ca^{2+} uptake into the SR show that the mean uptake rate in diseased myocardium is decreased by 36% (P=0.055) in homogenates from nonfailing and failing human hearts [13]. Similarily, Limas et al. [12] examined Ca^{2+} uptake rates in right ventricular biopsy specimen in patients with nonfailing myocardium and in those with dilated cardiomypathy and described a 49% decrease ($P<0.01$) in failing myocardium. In contrast to these findings, Movsesian et al. [15] observed no reduction in Ca^{2+} uptake into cardiac SR in patients with idiopathic dilated cardiomyopathy, which may be due to the fact that they performed their experiments on enriched SR preparations. Thus, differential enrichment of membrane components may have accounted for the results. Interestingly, there was no significant difference between SR Ca^{2+}ATPase activity in failing myocardium from patients with idiopathic dilated cardiomyopathy and with cardiomyopathy due to ischemic heart disease. This finding argues against specific changes of SR function depending on the underlying cause of heart failure.

The hearts examined in this study showed 55% lower SR Ca^{2+}ATPase mRNA levels in failing than in nonfailing myocardium. This difference was identical in each group, those with dilated and those with ischemic cardiomyopathy. Phospholamban mRNA levels were reduced in both types of failing left ventricular myocardium, but the decrease was more pronounced in idiopathic dilated cardiomyopathy (–45%, $P<0.001$) than in ischemic cardiomyopathy (–31%, $P<0.05$). Similar data have been obtained by Mercardier et al. [27] who detected a 48% decrease in relative SR Ca^{2+}ATPase mRNA levels in human failing hearts and by Linck et al. [29] who observed a decrease of SR Ca^{2+}ATPase mRNA levels by 50% in and of phosphoplamban mRNA levels by 30% in failing compared to nonfailing myocardium. Feldman et al. [28] reported an even more pronounced decrease in phospholamban mRNA levels (52%) in failing human hearts, performing polymerase chain reaction analysis with RNA from endomyocardial biopsies. Data obtained by Arai et al. [31] are also in accordance with these results but are difficult to compare, as they did not use nonfailing hearts as a control group but related their data to mRNA levels of atrial natriuretic factor as a marker for severity of

heart failure. According to their findings, there is a negative correlation between SR Ca^{2+}ATPase mRNA levels or phospholamban mRNA levels and atrial natriuretic factor mRNA levels in failing myocardium from patients with idiopathic dilated cardiomyopathy, ischemic heart disease, and primary pulmonary hypertension. In consequence, mRNA levels could be progressively decreased depending on the severity of heart failure, which exhibits a positive correlation to atrial natriuretic factor mRNA levels [49].

On the basis of findings in animal models one should expect that a decrease in SR Ca^{2+}ATPase mRNA levels and phospholamban mRNA levels is paralleled by a decrease in SR Ca^{2+}ATPase and phospholamban proteins [10, 32]. Interestingly, our data do now show this to be the case in human hearts. No significant differences in SR Ca^{2+}ATPase or protein levels were detected between failing and nonfailing myocardium. Our findings are in contrast to recent data of Hasenfuss et al. [13] and Studer et al [50]. Hasenfuss et al. [13] determined SR Ca^{2+}ATPase protein levels in nonfailing hearts and in end-stage failing hearts due to dilated and ischemic cardiomyopathy and found a significant reduction in SR Ca^{2+}ATPase levels in the failing myocardium by 36% ($P<0.02$) and by 32% ($P<0.05$) compared to nonfailing myocardium when SR Ca^{2+}ATPase levels were related to β-MHC levels. Within each group SR Ca^{2+}ATPase levels were distributed within a wide range. In our study we related protein levels of SR Ca^{2+}ATPase and of phospholamban to β-MHC protein levels as a marker for the amount of contractile proteins and to 5′-nucleotidase enzyme activity as a marker for the amount of membrane proteins. Although the relationship between mean values for SR Ca^{2+}ATPase and phospholamban levels in the examined groups was slightly altered, there were no significant differences between SR Ca^{2+}ATPase levels or phospholamban levels in nonfailing and in failing myocardium.

One major problem concerning the determination of phospholamban protein levels in failing and in nonfailing myocardium is that some antibodies recognize phosphorylated phospholamban with less affinity than nonphosphorylated. Thus one could argue that the missing difference concerning phospholamban protein level is due to impaired phosphorylation of phospholamban in the failing hearts. However, previous studies using the same antibody as in this report have shown that this specific antibody reacts similarily if not more strongly with the phosphorylated and nonsphorylated form and reacts similarily with the monomeric and the oligomeric form of phospholamban [51, 52]. The fact that the antibodies used in these studies are appropriate to detect quantitative differences was demonstrated both by western blot experiments in which different amounts of myocardial proteins were seperated to show that quantification is performed within the linear range, and by another recent study which – in contrast to this study – detected decreased left ventricular phospholamban and SR Ca^{2+}ATPase protein levels in a rat model of compensated hypertensive cardiomyopathy [53].

Our findings are in accordance with a recent report by Movsesian et al. [33] who also reported unchanged SR Ca^{2+}ATPase and phospholamban protein levels in failing human myocardium. These observations indicate that differences found in other studies may be either coincidental or, again, due to differences in membrane or vesicle preparation. In addition, one must be very critical on the relationship of data to β-MHC. Since hypertrophy of single myocardial cells has been observed in cardiomyopathy [54], it is likely that the β-MHC content per single myocyte is increased, and one dilutes SR Ca^{2+}ATPase per cell by relating measured concentrations to β-MHC. Therefore the data of the present study were related to several markers. No differences were observed in the different groups with any of the calculations, and it must therefore be concluded that in the hearts examined in this study steady-state levels of SR Ca^{2+}ATPase and phospholamban proteins are unchanged in end-stage heart failure due to dilated or ischemic cardiomyopathy.

Our data indicate that alterations in mRNA levels do not necessarily lead to equivalent changes at the level of proteins which may be due to changes in protein synthesis and degradation rate in the failing myocardium. Interestingly, Magid et al. [55] observed that development of heart failure in rabbits due to surgically induced aortic regurgitation leads to an increase in protein synthesis rate during the first 3 days after operation and a decrease in protein fractional degradation rate. These data favor the suggestion that protein synthesis and metabolism are altered in a rather complicated and unforseeable manner in heart failure. Thus one must emphasize that it is impossible to conclude uncritically from changes on the mRNA levels to protein levels or from protein levels to specific functions of enzymes or regulatory proteins. The data presented here provide evidence for a posttranscriptional or posttranslational modification or both of SR protein expression and function.

Since alterations in Ca^{2+} uptake function of the SR are apparently responsible for the disturbed diastolic Ca^{2+} handling and for the impaired relaxation of failing myocardium, but these changes are not mirrored by quantitative changes in SR proteins such as SR Ca^{2+}ATPase and phospholamban, additional regulatory factors or functional modifications of these proteins may be responsible for the pathological diastolic Ca^{2+} uptake into the SR. In this context a soluble inhibitor of SR Ca^{2+}-ATPase has been reported to be present in the ammonium fraction of cardiac and to slow skeletal muscle cytoplasm [56]. Subsequent studies have identified fractions enriched in actin as having inhibitory activity [57]. This inhibition was removed by muscle albumin [58]. Concerning functional modifications of SR Ca^{2+}-ATPase and phospholamban, the phosphorylation status of phospholamban is of special interest. While the native, nonphosphorylated form of phospholamban inhibits SR Ca^{2+}ATPase [22], this inhibitory effect of phospholamban is deactivated when the protein is phosphorylated in a cAMP dependent manner [24]. Thus, a reduced phosphorylation status of phospholamban in the intact myocardial cell during cardiac failure

is one candidate to produce SR dysfunction and diastolic Ca^{2+} handling disturbancy.

Since our data indicate that the amount of phospholamban is not changed, and since it has already been shown that there is no difference in cAMP-dependent protein kinase activity or cAMP-dependent phosphorylation of phospholamban by exogenous cAMP-dependent protein kinase and cAMP in failing myocardium [43], it appears likely that the key alteration is the reduction in cAMP levels in intact failing myocardium [20]. This reduction is due to the described desensitization of the β-adrenoceptor adenylate cyclase system [16–18, 20, 35, 59, 60] caused by a decrease in the number of β-adrenoceptors [16] and an increase in $Gi\alpha_2$ mRNA [34] and protein levels [17–19]. These features are typical for failing myocardium, and it has been shown that the characteristic changes in $Gi\alpha_2$ mRNA and protein levels are exhibited in the samples from failing hearts examined in this study. The functional relevance of the desensitization of the β-adrenoceptor adenylate cyclase system is supported by functional data of Mulieri et al. [61] who demonstrated a restauration of the reduced force-frequency relationship in failing myocardium following application of forskolin, the effect of which is not changed in heart failure [18, 59, 61]. Since the depressed force frequency relationship has been related to a depressed SR Ca^{2+}ATPase activity [13], these findings on a complete restauration of the force-frequency relationship by an elevation of cAMP levels with forskolin provide direct functional evidence for a key role of diminished cellular cAMP concentrations in failing hearts for the impairment of SR Ca^{2+}ATPase activity most likely due to a reduced phosphorylation status of phospholamban.

In summary, in the failing human myocardium a significant decrease in SR Ca^{2+}ATPase activity is observed which likely contributes to an altered SR Ca^{2+} handling. However, although mRNA levels of SR Ca^{2+}ATPase and phospholamban were significantly reduced in the failing myocardium, and there was an inverse correlation between $Gi\alpha_2$ mRNA and SR Ca^{2+}ATPase mRNA, no difference between failing and nonfailing myocardium was detected in SR Ca^{2+}ATPase or phospholamban protein levels. Changes in the steady-state protein content of SR proteins therefore do not contribute to the impaired Ca^{2+}ATPase activity. The dissociation between mRNA and protein levels provides evidence for a so far unknown posttransscriptional and posttranslational regulation of the proteins. The observed alterations are not dependent on the underlying cause of end-stage heart failure. Further studies are needed to examine functional disturbances of SR proteins, for example, an altered phosphorylation state of phospholamban, and the mechanism of the impaired gene expression of these proteins.

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