Expression of Dihydropyridine Receptor (Ca²⁺ Channel) and Calsequestrin Genes in the Myocardium of Patients with End-Stage Heart Failure

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Abstract

Cytoplasmic free calcium ions (Ca²⁺) play a central role in excitation–contraction coupling of cardiac muscle. Abnormal Ca²⁺ handling has been implicated in systolic and diastolic dysfunction in patients with end-stage heart failure. The current study tests the hypothesis that expression of genes encoding proteins regulating myocardial Ca²⁺ homeostasis is altered in human heart failure. We analyzed RNA isolated from the left ventricular (LV) myocardium of 30 cardiac transplant recipients with end-stage heart failure (HF) and five organ donors (normal control), using cDNA probes specific for the cardiac dihydropyridine (DHP) receptor (the α_1 subunit of the DHP-sensitive Ca²⁺ channel) and cardiac calsequestrin of sarcoplasmic reticulum (SR). In addition, abundance of DHP binding sites was assessed by ligand binding techniques ($n = 6$ each for the patients and normal controls). There was no difference in the level of cardiac calsequestrin mRNA between the HF patients and normal controls. In contrast, the level of mRNA encoding the DHP receptor was decreased by 47% ($P < 0.001$) in the LV myocardium from the patients with HF compared to the normal controls. The number of DHP binding sites was decreased by 35–48%. As reported previously, expression of the SR Ca²⁺-ATPase mRNA was also diminished by 50% ($P < 0.001$) in the HF group. These data suggest that expression of the genes encoding the cardiac DHP receptor and SR Ca²⁺-ATPase is reduced in the LV myocardium from patients with HF. Altered expression of these genes may be related to abnormal Ca²⁺ handling in the failing myocardium, contributing to LV systolic and diastolic dysfunction in patients with end-stage heart failure. (*J. Clin. Invest.* 1992. 90:927–935.) Key words: dihydropyridine receptor • calcium channel • calcium ATPase • calsequestrin • heart failure • human cardiac transplant

Introduction

Cytoplasmic free calcium ions (Ca²⁺) play critical second messenger functions in the regulation of numerous fundamental physiologic processes. The central role of Ca²⁺ in regulating muscle contraction is well established (1). Upon depolarization of the sarcolemma, extracellular Ca²⁺ enters the cells through voltage-gated calcium channels and triggers the release of Ca²⁺ from sarcoplasmic reticulum (SR).[1] The released Ca²⁺ binds to troponin C, which allows the myosin head to interact with actin to generate force. Reuptake of Ca²⁺ from the cytosol is mediated by the Ca²⁺-ATPase of the SR, in which Ca²⁺ is bound by calsequestrin and stored until the next cycle of excitation. Ca²⁺ is extruded from myocytes by the Na⁺/Ca²⁺ exchanger and the Ca²⁺-ATPase of the sarcolemma.

Abnormal Ca²⁺ handling observed in the ventricular myocardium from patients with end-stage heart failure has been suggested to play an important role in systolic and diastolic dysfunction observed in these patients (2). It has been reported that the SR Ca²⁺ uptake rate of myocardium obtained from patients with heart failure is diminished by 50% (3). These human studies are compatible with numerous animal studies of cardiac hypertrophy and failure, in which Ca²⁺ uptake rates by myocardial SR have been reported to be decreased (4–9, and reviewed in reference 10). The diminished rates of the SR Ca²⁺ uptake seem to be due to reduced expression of the Ca²⁺-ATPase gene both in animals (11–13) and humans (14, 15). In contrast, Movsesian et al. have reported that rates of the SR Ca²⁺ uptake as well as immunodetectable levels of the SR Ca²⁺-ATPase protein remained unchanged in the left ventricular (LV) myocardium from cardiac transplant recipients with idiopathic dilated cardiomyopathy as compared to normal controls (16–18).

There have been several reports about changes in expression of other SR protein genes. The level of mRNA encoding phospholamban, a regulatory protein of the SR Ca²⁺-ATPase, has been shown to be decreased in the hypertrophied right ventricles obtained from rabbits with pulmonary constriction (12) and in endomyocardial biopsies from patients with end-stage heart failure (19). Furthermore, the decreased level of mRNA encoding Ca²⁺-release channel (ryanodine receptor) was recently observed in the LV myocardium from patients with ischemic cardiomyopathy, but not from those with idiopathic dilated cardiomyopathy (20). On the other hand, the study conducted by Movsesian et al. (18) has demonstrated that immunodetectable levels of phospholamban, cardiac calsequestrin (a high-capacity, medium-affinity Ca²⁺-binding pro-

1. *Abbreviations used in this paper:* DHP, dihydropyridine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCM, hypertrophic cardiomyopathy; LV, left ventricular; PCR, polymerase chain reaction; SR, sarcoplasmic reticulum.

tein), or Ca^{2+}-release channel were not different between patients with idiopathic dilated cardiomyopathy and normal controls. These data suggest that there remain some controversies concerning the expression of these SR proteins in diseased myocardium.

Several studies have examined changes in the density of the Ca^{2+} antagonist receptor of the α_1 subunit of the dihydropyridine (DHP)-sensitive Ca^{2+} channel (Ca^{2+} channel) in the myocardium from cardiomyopathic hamsters (21–24), rats with pressure overload (25, 26) or infarction (27) and patients with idiopathic dilated cardiomyopathy (28, 29) and hypertrophic cardiomyopathy (30). In these diverse animal models and human diseases there have been conflicting findings on the abundance of DHP binding sites (reviewed in reference 31). It remains uncertain whether the LV expression of the cardiac DHP receptor (Ca^{2+} channel) is normal in human advanced heart failure.

There is a long-standing concern that the use of Ca^{2+} channel blockers contributes to the worsening of heart failure in patients with pre-existing left ventricular dysfunction (32, 33). The results from a series of recent clinical trials strongly support this concern (34–39, and reviewed in reference 40). Because Ca^{2+} channel blockers are widely used to treat angina pectoris or hypertension in patients with underlying LV dysfunction, it is important to gain more understanding of expression of the gene encoding the Ca^{2+} channel in the failing myocardium.

In order to characterize alterations in expression of the genes related to Ca^{2+} handling in human heart failure, we examined the levels of mRNAs encoding the cardiac DHP receptor, cardiac calsequestrin, and SR Ca^{2+}-ATPase in the LV myocardium from patients with end-stage heart failure. In addition, we quantitated the number of DHP binding sites by ligand binding assay.

Methods

Subjects. We analyzed LV RNA isolated from 30 patients (15) (27 male and 3 female, mean age of 40±15 yr [mean±SD]) with end-stage heart failure, undergoing cardiac transplant surgery at Brigham and Women's Hospital, Boston (Table I). The subjects included 12 patients with idiopathic dilated cardiomyopathy, 14 with coronary artery disease, 2 with hypertrophic cardiomyopathy, and 2 with congenital heart disease (1 with transposition of great vessels and 1 with an atrial septal defect: both of these patients had undergone prior cardiac surgery). Hemodynamic data obtained 0.3–20 (mean 4.7) mo before the transplant operations were as follows: cardiac index ($n = 26$), 1.8±0.5 liter/min·m²; mean pulmonary capillary wedge pressure ($n = 27$), 26±9 mmHg; LV end-diastolic pressure ($n = 15$), 27±9 mmHg; LV ejection fraction ($n = 8$), 27±19%. At the time of heart transplantation, hearts were explanted, quickly weighed, a piece (1–2 g) of myocardium was excised from the LV free wall, and immediately immersed in liquid nitrogen. Ventricular samples were obtained from areas that were not macroscopically scarred. Mean heart weight was 495±118 g. For control hearts, LV tissue was obtained from five organ donors who showed apparently normal cardiac function, but for whom a suitable transplant recipient could not be found. This study was approved by the Committee for the Protection of the Human Subjects from Research Risks at Brigham and Women's Hospital.

Isolation of the rat cardiac DHP receptor cDNA. In order to isolate a cDNA probe specific for the α_1 subunit of the cardiac Ca^{2+} channel (DHP receptor), we reverse-transcribed rat heart mRNA and performed polymerase chain reaction (PCR) using primers based on the published rabbit skeletal muscle DHP receptor cDNA (41): sense 18

mer = 5'-CCACGCTCCTGCAGTTCA-3'; anti-sense 30 mer = 5'-AGATGGTGTCGACCATGATGAGGGCGAACA-3'. The resultant band (570 bp) was subcloned into pGEM-3z and DNA sequence was determined by the dideoxy-chain termination method (42). Sequence comparison with the published rabbit cardiac DHP receptor cDNA revealed that this clone (p570) corresponds to the domain III-S5 to domain IV-S1 regions to the rabbit cDNA (43) and shows 90% and 95% sequence identity to the rabbit cDNA at nucleotide and amino acid levels, respectively (data not shown). This 570-bp clone was used to screen a rat heart cDNA library in λgt11 and the cDNA insert was subcloned into pBluescript. The clone pCDHP contained a 1.3-kb EcoRI insert, which had a 305-bp overlap at its 3' end with the p570 (44).

RNA preparation and analysis. Total cellular RNA was isolated from the ventricular tissues using the lithium chloride/urea method (45). The RNA was quantitated by spectrophotometry at 260 nm. The ratio of the absorbance at 260 nm to that at 280 nm was > 1.8 in all the samples. Aliquots (20 µg) of total cellular RNA were size-fractionated by electrophoresis on a 1.0% agarose/6% formaldehyde gel, and transferred to nitrocellulose filters. After baking at 80°C in a vacuum oven for 2 h, the filters were prehybridized in the solution mix containing 50% formamide, 5× SSC (1× = 0.15 M sodium chloride, 0.015 M sodium citrate), 5× Denhardt's solution, 0.2% SDS, 0.025 M sodium phosphate buffer (pH 6.5), and 250 µg/ml sonicated calf thymus DNA, at 42°C for at least 2 h. The filters were then hybridized with specific DNA probes in the same solution mix supplemented with 10% dextran sulfate at 42°C for 16 h. At the end of hybridization, the filters were washed serially and stringently with the final wash in 0.2× SSC/0.1% SDS at 55°C for 15 min for the DHP receptor probe and in 0.1× SSC/0.2% SDS at 65°C for 15 min for the other cDNA probes. DNA probes used in this study were as follows: (*a*) DHP receptor: a 1.3-kb EcoRI fragment isolated from a rat cardiac cDNA library (see above). This rat cardiac DHP receptor cDNA hybridizes to an mRNA species of ~ 8 kb in human heart RNA. Under the conditions used in this study for RNA blot analyses, this probe showed little or no detectable hybridization to ~ 15 kb mRNA species which may represent a smooth-muscle type DHP receptor (43, 44). (*b*) Cardiac calsequestrin: a 1.9-kb Eco RI fragment generated from a cDNA clone IC3A, containing the entire coding region and 3' untranslated region of the canine cardiac calsequestrin (46). (*c*) SR Ca^{2+}-ATPase: a 0.7-kb Pst I fragment corresponding to carboxyl terminal and 3' untranslated region of cDNA clone pCA, specific for the rabbit cardiac/slow twitch skeletal muscle SR Ca^{2+}-ATPase (47). (*d*) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): a 1.3-kb Pst I fragment generated from cDNA clone pUC-GAPDH13, containing the entire coding region and a part of the 3' untranslated region of the rat GAPDH (48). The cDNA probes were labeled by random priming method with [^{32}P]dCTP (3,000 Ci/mmol, New England Nuclear, Boston) and the ANP oligonucleotide with T4 polynucleotide kinase and ^{32}P-γATP (3,000 Ci/mmol, New England Nuclear).

Quantitation of mRNA. The filters were exposed at −80°C on X-ray films (X-OMAT, AR, Eastman Kodak Co., Rochester, NY) with intensifying screens (DuPont Co., Wilmington, DE). Relative amounts of mRNA species were determined by laser densitometry. No partially degraded RNA samples were used for densitometric analysis. Several exposures of X-ray films were obtained to ascertain the densitometric analysis was performed in the linear response range of the X-ray films. In order to confirm the accuracy of the densitometric analysis, phosphoimager analysis (Molecular Dynamics, Inc., Sunnyvale, CA) was performed in five patients. The same blots were also hybridized with GAPDH probe and the relative levels of GAPDH mRNA in each sample were determined by densitometry. Densitometric scores of specific mRNAs were normalized to that of mRNA encoding GAPDH as an internal control for RNA loading and transfer. We used GAPDH as an internal control because the levels of this mRNA species were not different between the patients and controls (patients: 1.00±0.15 vs. controls: 1.00±0.15, NS). GAPDH is constitutively expressed in most tis-

Table I. Clinical Characteristics of the Subjects

Case No.	Age	Sex	Diagnosis	CI	PCWP	LVEDP	LVEF	HW
	yr			*liter/min·m²*	*mmHg*	*mmHg*	*%*	*g*
1	35	M	DCM	2.5	21	26	18	420
2	18	M	DCM	1.4	29	NA	23	625
3	54	M	DCM	2	21	20	NA	440
4	14	M	DCM	1.4	15	8	24	300
5	18	M	DCM	NA	20	NA	NA	580
6	36	M	DCM	1.3	24	NA	NA	450
7	58	M	DCM	1.2	19	NA	NA	550
8	56	M	DCM	1.7	32	30	NA	500
9	NA	M	DCM	1.5	38	38	15	NA
10	23	M	DCM	0.9	42	32	NA	600
11	50	M	DCM	1.8	5	NA	NA	490
12	51	M	DCM	NA	NA	NA	NA	350
13	47	M	CAD	1.8	24	29	NA	405
14	44	M	CAD	2	23	NA	NA	620
15	55	M	CAD	NA	NA	NA	NA	577
16	33	M	CAD	2	34	41	NA	387
17	50	M	CAD	3.5	14	17	19	380
18	31	M	CAD	1.8	35	30	NA	375
19	48	M	CAD	1.6	38	16	20	460
20	58	M	CAD	1.3	28	NA	NA	560
21	50	M	CAD	2	28	NA	NA	470
22	54	M	CAD	1.4	34	27	NA	460
23	57	M	CAD	2.4	38	35	NA	595
24	54	M	CAD	1.8	26	NA	NA	370
25	24	F	CAD	1.4	24	NA	NA	NA
26	40	M	CAD	NA	NA	NA	NA	510
27	53	M	HCM	2.2	30	29	73	NA
28	19	F	HCM	1.5	13	NA	NA	440
29	20	M	CHD	2	18	24	NA	900
30	23	F	CHD	1.4	32	NA	NA	530
Mean	40			1.8	26	27	27	495
SD	15			0.5	9	9	19	118

Abbreviations: CAD, coronary artery disease (ischemic cardiomyopathy); CHD, congenital heart disease; CI, cardiac index; DCM, idiopathic dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; HW, heart weight; LVEDP, left ventricular end-diastolic pressure; LVEF, left ventricular ejection fraction; NA, not applicable; PCWP, pulmonary capillary wedge pressure.

sues and is the most widely accepted internal control in molecular biology literatures. Mean value of the normalized mRNA scores from the organ donors (normal controls) was arbitrarily set as 1.0 for each mRNA species.

DHP binding assay. In a separate series of experiments, sarcolemmal membranes were prepared as previously described (49) from the left ventricles of six organ donors, four patients with ischemic cardiomyopathy and two patients with idiopathic dilated cardiomyopathy. Specimens weighing 270–850 mg were thawed, minced and homogenized in 10 mM histidine, 10 mM $NaHCO_3$. After solubilization of contractile proteins in KCl, sarcolemmal membranes were sedimented at 40,000 g and resuspended in assay buffer (NaCl 150 mM, Tris 10 mM, pH 7.5) by Dounce homogenizer. The entire preparation was at 4°C. DHP binding was characterized by 18-point ^3H-(+)PN200-110 vs. unlabeled (+)PN200-110 competition binding curves conducted at 37°C for 30 min. Analysis of binding was computer-assisted nonlinear least-squares method (49).

Statistical analysis. All data are expressed as mean±SD. The statis-

tical significance of differences in mean values between the patients with end-stage heart failure and normal controls was assessed by the unpaired Student t test. Correlations between two variables were examined by linear regression analysis. Significance was accepted at $P < 0.05$ level.

Results

Characterization of the cardiac DHP receptor cDNA probe. Fig. 1 shows the partial nucleotide and amino acid sequences of the rat cardiac DHP receptor cDNA (pCDHP) used in the present study. Sequence comparison with the published rabbit cardiac DHP receptor cDNA has revealed that the pCDHP corresponds to the nucleotides 2411–3712, spanning from the domain II-S6 to the domain III-S6 regions of the cardiac DHP receptor (43). There was 92% (nucleotide) and 99.4% (amino acid) sequence identity between the pCDHP and the rabbit

DHP receptor cDNA in these regions (Fig. 1). This indicates that the pCDHP is a specific probe for the cardiac DHP receptor.

Developmental changes in expression of the cardiac DHP receptor and calsequestrin genes. In order to examine whether expression of the cardiac DHP receptor and calsequestrin genes undergoes developmental regulation, we performed Northern blot analysis using RNA samples isolated from human fetal ventricles (17 and 19 wk of gestation) and normal adult ventricles. The DHP receptor cDNA probe detected a single mRNA species of ~ 8 kb in all the human ventricular samples (Fig. 2 A). The level of expression of the cardiac DHP receptor mRNA was much lower in the fetuses compared to the normal adults. Similarly, expression of the cardiac calsequestrin mRNA (Fig. 2 B) and that of the SR Ca^{2+}-ATPase (15) were much lower in the fetal ventricles. In contrast to the rat heart, in which expression of the SR Ca^{2+}-ATPase and cardiac calsequestrin was shown to be regulated under different mechanisms during the fetal stage (50), our data suggest that expression of these SR protein genes seems to be regulated in parallel in the fetal human ventricles as shown in fetal rabbit hearts (51).

Verification of the densitometric analysis by phosphoimager. In order to verify whether the densitometric analysis we used to quantitate the levels of specific mRNA species in this study is accurate in measuring the DHP receptor mRNA (a low abundance message), we directly compared the results of densitometry of a Northern blot to those obtained by phosphoimager analysis of the same blot. As shown in Fig. 3 (*bottom*), there was an excellent agreement between the densitometric scores and the counts obtained by phosphoimager ($r = 0.998$, $P < 0.001$). We elected not to use dot blot analysis to quantitate the DHP receptor mRNA because this method would not differentiate specific hybridization signals of the DHP receptor mRNA from nonspecific hybridization signals to 28S ribosomal RNA (see Fig. 2 A).

Expression of the Ca^{2+} regulatory protein genes in the failing human myocardium. To determine whether expression of the Ca^{2+} regulatory protein genes is altered in the failing human myocardium, we performed RNA blot analysis using RNA isolated from the left ventricles of organ donors (Controls) and patients with end-stage heart failure (CHF). Fig. 3 shows the results of representative RNA blot analyses using the specific DNA probes. The cDNA probe corresponding to cardiac DHP receptor (the α_1 subunit of the DHP-sensitive Ca^{2+} channel) hybridized with a single mRNA species of ~ 8 kb in all the cases studied. The LV level of the DHP receptor mRNA was decreased in the patients with end-stage heart failure, due to both idiopathic dilated cardiomyopathy (*DCM*) and coronary artery disease (*CAD*), as compared with the normal con-

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          2411            2633                              3404            3710
           |               |                                 |               |
 pCDHP   ┌──────────────────────────────────────────────────────────────────────┐
         │  II-S6          III-S1 III-S2 III-S3 III-S4       III-S5         III-S6 │
         └──────────────────────────────────────────────────────────────────────┘
         <───────────────────>                          <────────────────────────>
                   A                                                 B
         └────────┘
          100 bp
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A

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gtg atg tat gat ggg atc atg gct tat ggc gac ccc tct
... ... ... ... ... ... ... ... ... ... .g. ... ...
 V   M   Y   D   G   I   M   A   Y   G   D   P   S
 -   -   -   -   -   -   -   -   -   G   -   -

ttt cca ggg atg tta gtc tgt att tac ttc atc atc ctc
... ... ... ... ... ... ... ... ... ... ... ... ...
 F   P   G   M   L   V   C   I   Y   F   I   I   L
 -   -   -   -   -   -   -   -   -   -   -   -

ttc atc tgt gga aat tat atc cta ctg aat gtg ttc ttg
... ... ... ... ... ... ... ... ... ... ... ... ...
 F   I   C   G   N   Y   I   L   L   N   V   F   L
 -   -   -   -   -   -   -   -   -   -   -   -

gcc att gcg gtg gac aac ctg gct gat gcg gag agc ctg
... ... ..g ... ... ... ... ... ... ..t ... ... ..t
 A   I   A   V   D   N   L   A   D   A   E   S   L
 -   -   -   -   -   -   -   -   -   -   -   -

acc tct gcc caa aaa gag gaa gaa gaa gag aag gag aga
..t ... ... ... ..g ..a ..g ... ... ... ... ... ...
 T   S   A   Q   K   E   E   E   E   E   K   E   R
 -   -   -   -   -   -   -   -   -   -   -   -

aag aag ctg gcc agg act gcc agc cc
... ... ... ... ... ... ... ... ...
 K   K   L   A   R   T   A   S   P
 -   -   -   -   -   -   -   -
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B

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atg ttc gcc tgc att ggg gtc cag ctc ttc aag gga aag
... ... ... ... ..c ..a ... ... ... ... ... ..g ...
 M   F   A   C   I   G   V   Q   L   F   K   G   K
 -   -   -   -   -   -   -   -   -   -   -   -   -

ctc tat acc tgt tcg gat agt tcc aaa cag acg gag gca
..g ..c ... ... ..a ..c ... ... ... ... ..t ... ..t
 L   Y   T   C   S   D   S   S   K   Q   T   E   A
 -   -   -   -   -   -   -   -   -   -   -   -   -

gaa tgc aag ggt aac tat ata aca tac aaa gac gga gaa
... ... ... ..,. ..c ..c ..c ... ... ... ..t ... ..g
 E   C   K   G   N   Y   I   T   Y   K   D   G   E
 -   -   -   -   -   -   -   -   -   -   -   -   -

gtt gac cac ccc att atc cag cct cga agt tgg gag aac
... ... ..t ... ... ..c ... ..g ..c ..c ... ... ...
 V   D   H   P   I   I   Q   P   R   S   W   E   N
 -   -   -   -   -   -   -   -   -   -   -   -   -

agc aag ttc gac ttt gac aat gtt ctg gca gcc atg atg
... ... ... ... ... ... ... ..c ..c ... ... ... ...
 S   K   F   D   F   D   N   V   L   A   A   M   M
 -   -   -   -   -   -   -   -   -   -   -   -   -

gcc ctc ttt acc gtc tcc acc ttc gag ggg tgg cca gag
... ... ..c ..t ... ... ... ... ... ..c ... ... ...
 A   L   F   T   V   S   T   F   E   G   W   P   E
 -   -   -   -   -   -   -   -   -   -   -   -   -

ctg ctg tac cgc tcc att gac tcc cac aca gaa gac aag
... ... ... ... ... ..c ... ... ... ..g ... ... ...
 L   L   Y   R   S   I   D   S   H   T   E   D   K
 -   -   -   -   -   -   -   -   -   -   -   -   -

ggt ccc atc tac aac tat cgt gtg gag atc tcc
..c ..t ... ... ... ..c ..a ... ... ... ...
 G   P   I   Y   N   Y   R   V   E   I   S
 -   -   -   -   -   -   -   -   -   -
```
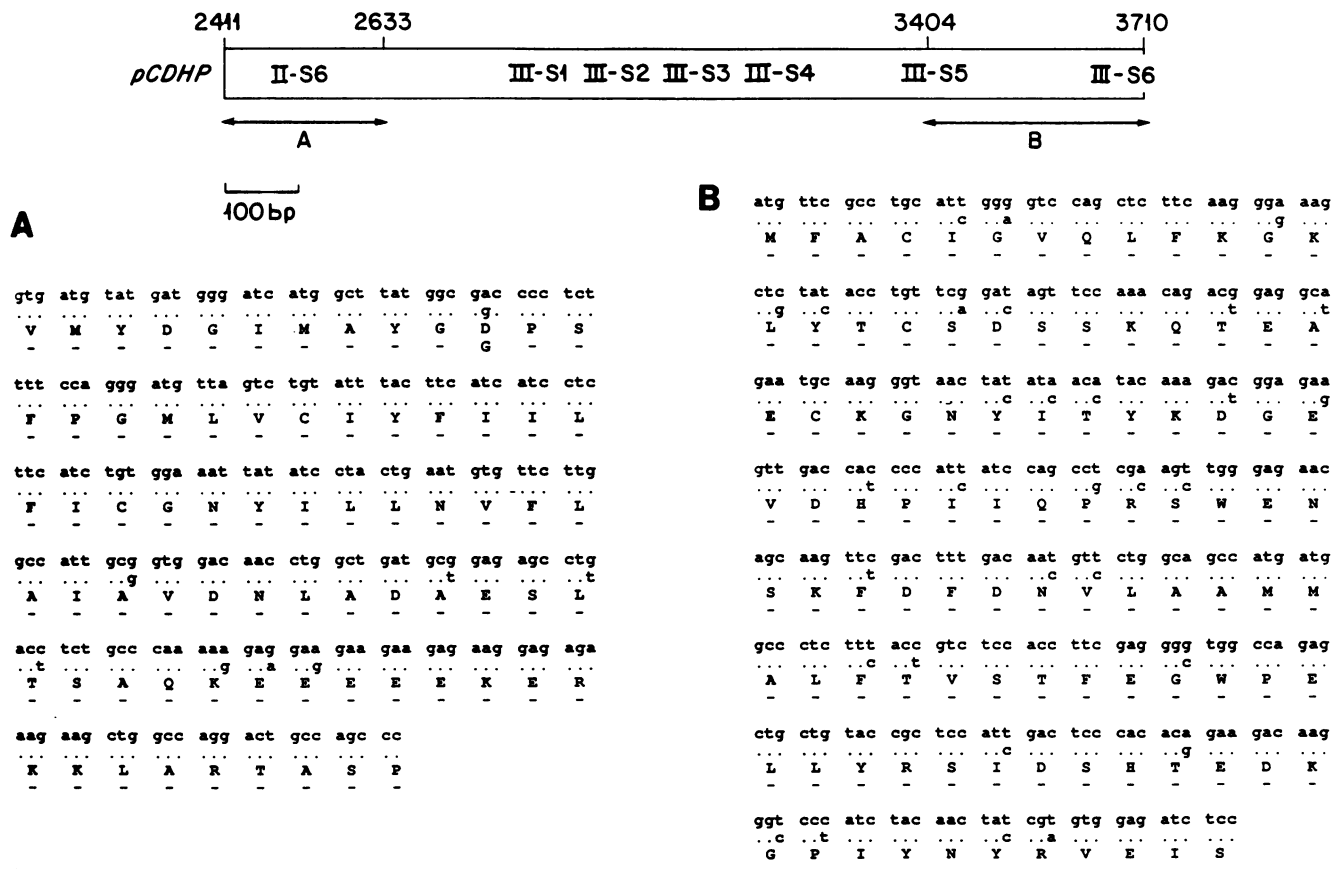
Figure 1. Characterization of the rat cardiac DHP receptor cDNA clone (pCDHP). Sequence comparison with the published rabbit cardiac DHP receptor cDNA has revealed that pCDHP corresponds to nucleotides 2411–3712, which spans from the domain II-S6 to the domain III-S6 regions of the rabbit cardiac DHP receptor (43). There was 92% (nucleotide) and 99.4% (amino acid) identity between the pCDHP (*first and third rows*) and the rabbit cardiac DHP receptor cDNA (*second and fourth rows*) for the sequenced portions. Sequence identity was shown in dots (for nucleotide) or dashes (for amino acid).
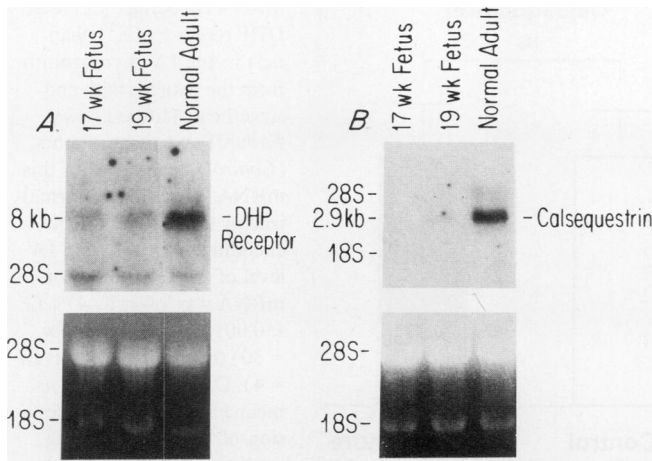
Figure 2. Developmental changes in expression of the cardiac DHP receptor and calsequestrin genes. (*A*) The DHP receptor cDNA probe detected an mRNA species of ~ 8 kb in all the human ventricular RNA samples. The level of expression of this mRNA was much lower in the fetal ventricles compared to the normal adults. (*B*) Similarly, ventricular expression of the cardiac calsequestrin mRNA was much lower in the fetuses. The images of ethidium bromide-stained 28S and 18S ribosomal RNAs are shown as an internal control for RNA loading.

trols (see below for quantitative analysis). The SR Ca^{2+}-ATPase probe detected a single mRNA species of ~ 4 kb in all the subjects. The level of mRNA encoding the SR Ca^{2+}-ATPase was also very low in the LV myocardium from the cardiac transplant recipients, regardless of the etiology of heart failure. The cardiac calsequestrin probe hybridized to a single mRNA band of ~ 2.9 kb. In contrast to the DHP receptor and SR Ca^{2+}-ATPase, the level of the calsequestrin mRNA remained unchanged in the LV myocardium from the patients. The cDNA probe for GAPDH, a constitutively expressed gene, hybridized with an mRNA species of ~ 1.6 kb. The level of the mRNA encoding this glycolytic pathway enzyme was similar both in controls and in the patients with end-stage heart failure.

Fig. 4 shows the results of densitometric determination of the relative abundance of each mRNA species and Fig. 5 shows the data from each individual patient in different etiologic categories. The densitometric score for each mRNA was normalized by the relative abundance of GAPDH as an internal control for loading and transfer. The mean value of the normalized scores of the organ donors (normal controls) was arbitrarily set at 1.0. The mean LV level of mRNA encoding the DHP receptor decreased by 47% ($P < 0.001$) in the patients with end-stage heart failure as compared to that of the organ donors (Fig. 4, *left*). There was no significant difference in the level of the DHP receptor mRNA between the patients with dilated cardiomyopathy (0.48±0.12) and those with ischemic cardiomyopathy (0.53±0.11) (Fig. 5, *left*). The level of the DHP receptor mRNA appears decreased in patients with end-stage hypertrophic cardiomyopathy (*HCM:* mean 0.73) and congenital heart disease (*CHD:* mean 0.53), although the small numbers of patients in these groups do not allow us to perform the subgroup analysis.

In contrast to that of the DHP receptor mRNA, expression of the cardiac calsequestrin mRNA remained unchanged in the end-stage heart failure patients (Fig. 4, *right*), regardless of the
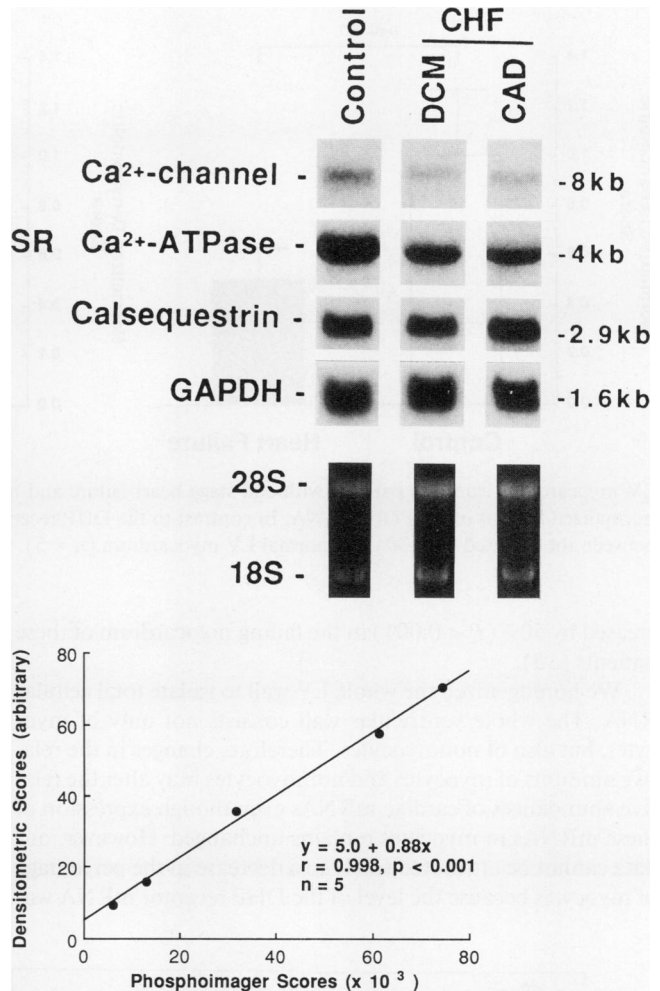

Figure 3. (*Top*) Results of representative RNA blot analysis using specific DNA probes. Samples loaded on the gel were 20 µg of total cellular RNA isolated from normal controls (*Control*) and patients with heart failure (*CHF*) due to idiopathic dilated cardiomyopathy (*DCM*) or coronary artery disease (*CAD*). The cardiac DHP receptor (Ca^{2+} channel) probe hybridized with an mRNA species of ~ 8 kb in all the samples. The SR Ca^{2+}-ATPase probe detected an mRNA species of ~ 4 kb in all the subjects. The LV expression of mRNAs encoding the DHP receptor and SR Ca^{2+}-ATPase was much lower in the CHF patients than those in the normal controls. The cardiac calsequestrin probe hybridized to a single mRNA band of ~ 2.9 kb. In contrast to the DHP receptor and SR Ca^{2+}-ATPase, the level of the calsequestrin mRNA remained unchanged in the LV myocardium from the patients. The mRNA encoding GAPDH was ~ 1.6 kb and the level of GAPDH mRNA was similar between the normal and failing LV myocardium. The images of ethidium bromide-stained 28S and 18S ribosomal RNAs were also shown as an internal control for RNA loading. Some of the hybridization signals presented here are overexposed for a better photographic reproduction. The densitometric analysis (shown in Figs. 4 and 5) was done in different exposures to ensure the linear response range of the X-ray films. (*Bottom*) Verification of the densitometric analysis by phosphoimager. There was an excellent agreement between the densitometric scores and the counts obtained by phosphoimager ($r = 0.998$, $P < 0.001$) of the cardiac DHP receptor mRNA signals.

etiology of heart failure (*DCM:* 1.02±0.21, *CAD:* 1.01±0.27, P = NS) (Fig. 5, *right*). In consensus with the previous report (14), the mean level of the SR Ca^{2+}-ATPase mRNA was de-

Ca²⁺ Channel

p<0.001

Control   Heart Failure

Calsequestrin
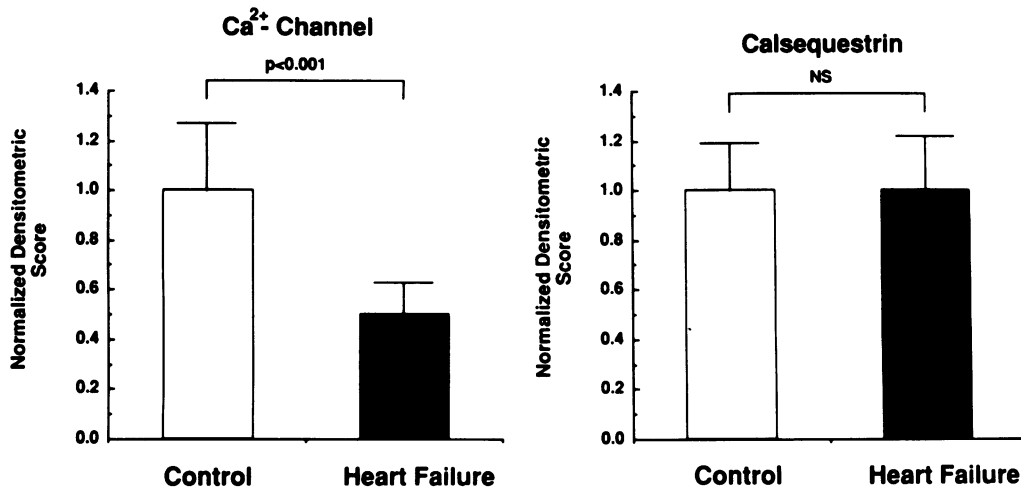
NS

Control   Heart Failure

Figure 4. (Left) The levels of mRNA encoding the cardiac DHP receptor (Ca²⁺ channel) in the LV myocardium from the patients with end-stage heart failure (*Heart Failure*) and organ donors (*Control*). The levels of this mRNA species were normalized by those of the mRNA encoding GAPDH. The LV level of the DHP receptor mRNA was lower by 47% ($P < 0.001$) in the patients ($n = 30$) than in the controls ($n = 4$). Data are expressed as mean±SD. (*Right*) Expression of the gene encoding cardiac calsequestrin in the LV myocardium from the patients with end-stage heart failure and normal controls. The levels of the mRNA encoding this protein were also normalized by that of GAPDH mRNA. In contrast to the DHP receptor, the level of cardiac calsequestrin mRNA did not differ significantly between the diseased ($n = 30$) and normal LV myocardium ($n = 5$).

creased by 50% ($P < 0.001$) in the failing myocardium of these patients (15).

We homogenized the whole LV wall to isolate total cellular RNA. The whole ventricular wall consists not only of myocytes, but also of nonmyocytes. Therefore, changes in the relative amounts of myocytes and nonmyocytes may alter the relative abundances of cardiac mRNAs even though expression of these mRNAs in myocytes remains unchanged. However, our data cannot be attributed solely to a decrease in the percentage of myocytes because the level of the DHP receptor mRNA was

significantly depressed even after corrected for the level of the calsequestrin mRNA (a myocyte-specific gene product) in each patient, also regardless of the etiology of heart failure (*Control:* 0.96±0.20; *DCM:* 0.49±0.13; *CAD:* 0.55±0.14, $P < 0.001$).

To determine whether end-stage heart failure is associated with alterations in the number of DHP binding sites, ligand binding experiments were conducted in a separate series of normal and congestive heart failure ventricles. A representative PN200-110 binding curve is shown in Fig. 6. Ligand binding parameters are summarized in Table II. The dissociation constant for (+)-PN200-110 binding to normal tissue was 265±46 pM and was unchanged in heart failure tissue (233±32 pM). However, the number of DHP binding sites in congestive heart
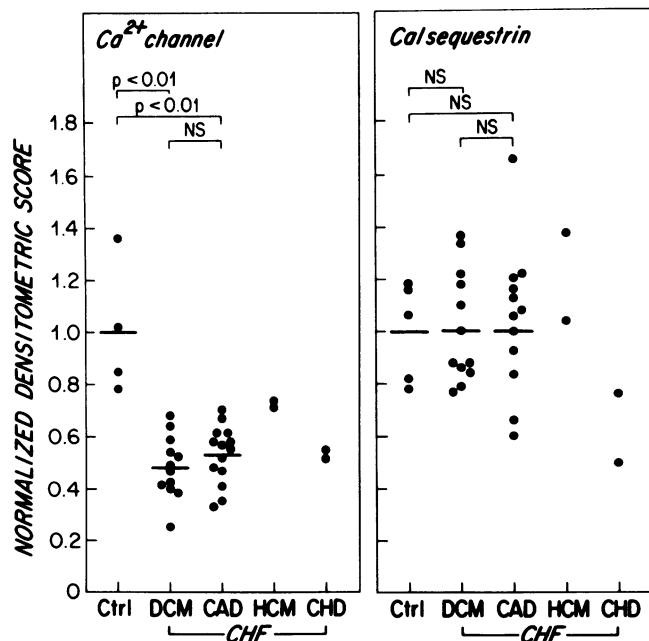

Figure 5. The levels of mRNAs encoding the cardiac DHP receptor (Ca²⁺ channel) (*left*) and calsequestrin (*right*) in individual patients classified by the underlying etiologies of heart failure. Abbreviations: Ctrl, normal control (organ donor); CHF, congestive heart failure; DCM, idiopathic dilated cardiomyopathy; CAD, ischemic cardiomyopathy; CHD, congenital heart disease; HCM, hypertrophic cardiomyopathy.
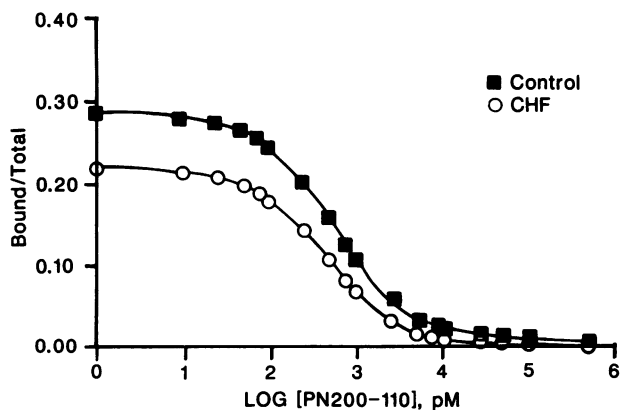

LOG [PN200-110], pM

Figure 6. Dihydropyridine (DHP) binding to ventricular membranes. For these typical experiments, a fixed concentration of ³H-(+)-PN200-100 (near the K_D) and graded concentrations of unlabeled (+)-PN200-100 were incubated with ventricular membranes. Bound/total ligand is plotted on the ordinate; total [PN200-100], expressed in picomolar, on the abscissa. Binding to membranes from control heart are shown in squares and from a heart with LV failure in circles. The decrease in the PN200-100 binding sites is evident by the downward displacement of the curve for the failing LV myocardium. Equilibrium binding isotherms demonstrated that binding was > 90% specific at PN200-100 concentrations near the K_D.

Table II. Ligand Binding Parameters and DHP Binding Site Numbers

	K_D	No. of DHP binding sites	No. of DHP binding sites	No. of DHP binding sites
	pM	fmol/mg protein	fmol/mg wet wt	fmol/μg DNA
Normal	265±46	88±5	5.70±0.35	18.9±3.6
CHF	233±32	60±9*	3.72±0.40[†]	9.8±1.9*

Abbreviations used as: CHF, congestive heart failure; K_D, dissociation constant. $n = 6$ for normal and for CHF.
* $P = 0.02$ compared to normal. [†] $P < 0.01$ compared to normal.

failure tissue was reduced to 68% of that in normal tissue when the number of DHP binding sites was normalized to fmol/mg of noncollagen protein. Normalizing the amount of membrane proteins in hypertrophied and failing hearts is sometimes problematic because of cellular hypertrophy and interstitial fibrosis that are often found in these end-stage hearts. Accordingly, the number of DHP binding sites was also normalized per milligram of wet weight heart tissue and per microgram of DNA. Regardless of the method of normalization, the number of DHP binding sites was significantly reduced by 35–48% (Table II).

To examine whether expression of the genes encoding the Ca^{2+} regulatory proteins is regulated in a coordinated manner in the failing human myocardium, we analyzed correlative relationships among the levels of expression of these genes. There were no significant correlations among the levels of mRNAs encoding the DHP receptor, SR Ca^{2+}-ATPase or cardiac calsequestrin in the LV myocardium from the patients (data not shown).

Discussion

The present study demonstrates that the level of mRNA encoding DHP receptor in the failing adult myocardium was significantly diminished compared with normal controls. As reported previously (15), the level of the SR Ca^{2+}-ATPase mRNA was also reduced in these failing ventricles. This diminished expression of these genes in the failing myocardium is not due to a generalized depression of cardiac gene expression, because the level of cardiac calsequestrin mRNA was not altered and that of atrial natriuretic peptide mRNA was markedly augmented in these ventricles (15).

The current data demonstrated that expression of the α_1 subunit gene for the slow Ca^{2+} channel was diminished in the LV myocardium from patients with end-stage heart failure. This is the subunit that forms the actual pore for Ca^{2+} entry into the heart cell and is the target for clinically used Ca^{2+} channel blockers (52). There was no change in ligand binding affinity, suggesting that at least the binding domain of the gene product is unaltered. Regardless of the method of normalization, the number of DHP binding sites is significantly reduced. The signal for decreased expression of the Ca^{2+} channel α_1 subunit gene is uncertain, but may possibly be related to the high plasma catecholamine concentrations found in patients with end-stage heart failure that has been demonstrated to down-regulate adrenergic receptors (53). Marsh (54) has demonstrated in a myocyte culture system that β-adrenergic stimulation can produce concomitant down-regulation of β-adrenergic receptors and DHP binding sites.

The observation that expression of DHP receptor mRNA is decreased in end-stage heart failure should not be interpreted to be of pathogenic importance, because only the terminal stage of the disease was examined. However, the decrease in abundance of DHP receptors in this setting may have clinical implications regarding further depression of excitation–contraction coupling by the clinical use of Ca^{2+} channel blockers. This may explain, at least in part, the deleterious effects of Ca^{2+} channel blockers in patients with congestive heart failure (32–40).

In contrast to our present observations, Rasmussen et al. (29) reported that the number of DHP binding sites was not altered in ventricles of patients with idiopathic dilated cardiomyopathy. Although the reasons for the differences between these two studies are not clear, one factor may be differences in patient groups studied. Idiopathic dilated cardiomyopathy is probably not a disease of a single cause but of multiple etiologies. No prior report is available concerning DHP binding sites in patients with ischemic cardiomyopathy. It should be noted that even in well-characterized animal models, an increase, decrease, or no change in the number of DHP binding sites has been reported at various stages of LV hypertrophy and failure (21–27, 31). The current study provides new data on DHP receptor mRNA at a well-defined point in human LV failure. These data are not directly comparable to, and stand apart from, previous reports in animals.

Two of the hearts in the current study had end-stage failure associated with the hypertrophic cardiomyopathy (HCM) phenotype. It is of note that ventricles from these two hearts showed the highest levels of DHP receptor mRNA (normalized scores 0.72 and 0.73) among the patient groups. Atrial tissue from human HCM patients had previously been reported to have increased DHP binding sites at an earlier stage of the disease (30). Thus, it is possible that in HCM the number of DHP binding sites may be increased early in the course of the disease and that the number diminished less in the end-stage of the disease than for heart failure of other etiologies. It should be noted that we examined expression of DHP receptor mRNA in LV myocardium, while Wagner et al. (30) examined DHP binding in atrial tissue. Therefore, the differences observed may also be due to those between the atrium and ventricle.

Decreased myocardial expression of the SR Ca^{2+}-ATPase mRNA was observed in human heart failure (14, 15) and animal models of cardiac hypertrophy (11–13). These data are compatible with the diminished SR Ca^{2+} uptake rates shown in both human heart failure (3) and animal models of myocardial hypertrophy and failure (4–10, 11, 13), although Movsesian et al. (16–18) reported that there were no differences in either the SR Ca^{2+} uptake rates or immunodetectable level of the SR Ca^{2+}-ATPase protein between patients with idiopathic dilated cardiomyopathy and normal controls.

A formal (though unlikely) possibility exists that expression of DHP receptor and SR Ca^{2+}-ATPase mRNAs is not reduced relative to other transcripts within cardiomyocytes. Rather, the appearance of a reduction may be created by loss of myocytes relative to non-myocytes cells within the failing hearts, a change that would maintain the pool of ribosomal RNA and GAPDH mRNA against an apparent decline or "dilution" of muscle-specific transcripts. This argument is made much less likely by the observation that calsequestrin mRNA

remained constant when normalized against ribosomal RNA or GAPDH mRNA. However, a loss of myocytes relative to non-myocytes and increased expression of calsequestrin mRNA within myocytes (with no change in expression of DHP receptor and SR Ca^{2+}-ATPase mRNAs within myocytes) also could account for the empirical data. We believe that this is highly unlikely for the following reasons. First, others also have observed that the level of Ca^{2+}-ATPase mRNA, corrected by myosin heavy chain mRNA, is decreased in patients with heart failure (14) and there is no evidence that the level of the myosin heavy chain mRNA is increased in the failing hearts (14, 19). Second, in order to "dilute" a muscle specific transcript by 50%, a magnitude of decline that we observed here, the cellular mass (not the number) of nonmyocytes should occupy at least half of the myocardium in the failing hearts. This is not what we observed in these hearts.

Lack of significant correlations among the levels of mRNAs encoding the DHP receptor, SR Ca^{2+}-ATPase, and cardiac calsequestrin in individual patients suggest that expression of these genes is regulated independently in the failing LV myocardium. This contrasts with developmental regulation in which the fetal expression is low for all three genes. Recently, Lompre et al. (50) reported that in the fetal and aged rat, ventricular expression of SR Ca^{2+}-ATPase was diminished but that of cardiac calsequestrin remained unchanged. In rabbit skeletal muscle, denervation has been observed to change the composition of the SR Ca^{2+}-ATPase and calsequestrin in the same manner as we observed in the LV myocardium from patients with end-stage heart failure (55). Further studies are needed to elucidate the mechanisms and functional significance of the alteration in the relative abundance of mRNAs encoding these two SR proteins.

Some limitations of the present study should be noted. First, we measured DHP binding sites in sarcolemmal membranes, not the DHP receptor protein itself. An antibody specific to the human cardiac DHP receptor protein (none available currently) will be necessary to make a direct measurement of the level of the α_1 subunit of the Ca^{2+} channel. Second, because the ligand binding assay and mRNA measurement were independently performed, only three patients had both measurements done simultaneously, a number too small to make a correlation between the two parameters in individual patients. Therefore, at present we are unable to conclude that expression of DHP receptor is regulated at pretranslational level in individual patients. Perhaps an animal study with a detailed kinetics at mRNA and protein levels (56) would be a more suitable system to answer such a question. Third, we used organ donor hearts as normal controls. They are not matched to heart failure patient groups with respect to age, sex or other clinical parameters. Although these hearts appeared grossly normal, they were exposed to endogenous, and in some cases, low dose exogenous catecholamines in the patient prior to harvest. Our control subjects did not have major cardiac abnormalities, or no significant ventricular expression of atrial natriuretic peptide was observed in any case (15).

Finally, it is not possible to determine from this study whether the alterations in the DHP receptor gene expression are pathogenic for LV dysfunction in patients with end-stage heart failure, or are secondary processes induced by heart failure. Although it is likely that the latter is true, the altered expression of the genes encoding the DHP receptor and other Ca^{2+} regulatory proteins may lead to disturbances in Ca^{2+} ho-

meostasis in failing myocardium and contribute to impairment of systolic and diastolic function in these patients (57).

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