Subunit Expression of the Cardiac L-Type Calcium Channel Is Differentially Regulated in Diastolic Heart Failure of the Cardiac Allograft

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Background—Left ventricular diastolic dysfunction is a major cause of cardiac allograft failure. Multimeric L-type calcium channels (α1-, α2δ-, and β-subunits) are essential for excitation/contraction coupling in the heart. Their gene expression was studied in allografts that developed diastolic heart failure.

Methods and Results—mRNA levels of calcium channel subunits were measured by competitive reverse transcriptase–polymerase chain reaction in microbiopsy samples from the interventricular septum. Size and tissue variabilities between biopsy samples were assessed by determination of cardiac calsequestrin mRNA levels. In the cardiac allografts studied, mRNA levels in microbiopsy samples were considered to represent left ventricular gene expression, because septal and left ventricular gene expression in Northern blots was equivalent, and left ventricles contracted homogeneously. Biopsy samples (n=72) were taken from allografts with normal left ventricular end-diastolic pressure (LVEDP; 8 to 13 mm Hg; n=30), moderately elevated LVEDP (14 to 18 mm Hg; n=26), and elevated LVEDP (19 to 28 mm Hg; n=16). Increased LVEDP was related to slowed diastolic relaxation determined by the time constant τ (r²=0.86), whereas systolic performance (dP/dt; ejection fraction) was preserved. With increasing LVEDP, mRNA levels of the pore-forming α1c-subunit (n=15) and of the regulatory α2δ-subunit (n=17) remained unchanged but decreased exponentially (r²=−0.83) for the regulatory β-subunit (n=40). Compared with cardiac allografts with normal LVEDP (n=15), β-subunit mRNA level was reduced by 75% at elevated LVEDP (n=9; P=0.012). In an explanted, diastolically failing cardiac allograft, β-subunit expression was reduced correspondingly by 72% and 76% on the mRNA level in septal and left ventricular myocardium and by 80% on the protein level.

Conclusions—The downregulated expression of the calcium channel β-subunit might contribute to altered calcium handling in diastolically failing cardiac allografts. (Circulation. 1999;100:155-163.)

Key Words: calcium channels • diastolic heart failure

In the heart, excitation/contraction coupling depends on L-type calcium channels. Cardiac calcium channels are membranous multimers consisting of the pore-forming α1c-subunit and the regulatory α2δ- and β-subunits.1–3 The α1c-subunit contains receptor sites for the calcium channel blocker and determines the basic electrophysiological properties.1 β-Subunits play important functional roles in the formation and stabilization of calcium channels: they enhance the density of dihydropyridine binding sites, increase dihydropyridine-sensitive barium inward currents, accelerate current activation and inactivation, and shift the half-maximal steady-state activation and inactivation to hyperpolarizing potentials.4–6 β-Subunits are also involved in the β-adrenergic signal transduction pathway by direct phosphorylation through the cAMP-dependent protein kinase.7 Four different β-subunit coding genes have been identified (β1,2,3).8 In human heart, mRNA expression of β1- and β3-subunit transcripts3,9 and protein expression of a β3-subunit10 have been demonstrated. The 3 regulatory α2δ-subunits have been cloned. They shift voltage dependence of channel activation and inactivation in a hyperpolarizing direction and accelerate current inactivation kinetics.11 In human heart, expression of α2δ1 has been demonstrated.2

In human heart failure, either reduced or unchanged α1c-subunit mRNA levels12,13 or numbers of dihydropyridine binding sites12,14 were reported. These variabilities are possibly due to heterogeneous patient groups with differences in the underlying heart disease and cardiac systolic or diastolic performance.15 However, heart transplant recipients represent a rather homogeneous patient collective in which some of the cardiac allografts develop diastolic heart dysfunction in the presence of preserved systolic function.16,17 To study cardiac calcium channel subunit gene expression in diastolic dysfunction, mRNA levels were determined in microbiopsy samples...
taken from cardiac allografts with and without diastolic dysfunction. Calcium channel subunit mRNA levels were normalized to the cardiac calsequestrin mRNA level to account for variations in biopsy size and tissue composition. Calsequestrin is a calcium storage protein of the sarcoplasmic reticulum with unchanged expression on mRNA and on the protein level in heart failure.\textsuperscript{12,18} It is predominantly expressed in cardiomyocytes and to a lesser extent in other tissues.\textsuperscript{19} Therefore, it can be used as a marker for overall gene expression\textsuperscript{20} and can provide an assessment of cardiomyocyte content in biopsy. This new approach of mRNA expression assessment in microbiopsy samples was used to characterize calcium channel gene expression changes in cardiac allografts with and without diastolic dysfunction.

Methods

Patients

Seventy-two patients (12 women aged 20 to 72 years; 60 men aged 23 to 70 years; mean age, 52 years) were studied 3 months to 9 years after orthotopic heart transplantation. Immunosuppressive treatment was accomplished with cyclosporine or FK 506, prednisone, and azathioprine. The 72 patients examined had no biopsy evidence of allograft rejection greater than or equal to class Ib at time of biopsy.\textsuperscript{21} One patient (patient 73) who showed rejection class II was treated with high-dose prednisone and underwent repetitive biopsies. Thirty-two patients had experienced previous episodes (≥2 episodes) of moderate to severe allograft rejection. Forty-six patients were treated with diltiazem.

Interventricular septum or left ventricular myocardium probes were obtained from explanted normal hearts not transplanted for technical reasons and from an explanted diastolically failing allograft. The study was approved by the local institutional review committee.

Cardiac Catheterization

Transplant recipients (n=72) underwent cardiac catheterization as part of their routine annual postoperative clinical evaluation. Aortic and left ventricular pressures were determined by fluid-filled catheters connected to Statham transducers. Left ventricular isovolumic relaxation was determined by the time constant \(\tau\).\textsuperscript{22} End-diastolic volume (EDV) and ejection fraction (EF) were calculated by the centerline method.\textsuperscript{23} Three endomyocardial biopsy samples were harvested for histological assessment, and 1 microbiopsy sample was harvested for study purposes.

mRNA Isolation and Northern Blot Analysis

RNA was isolated with the Trizol reagent\textsuperscript{24} from the interventricular septum and the left ventricular wall. mRNA was separated from total tissues.\textsuperscript{19} Therefore, it can be used as a marker for overall gene expression\textsuperscript{20} and can provide an assessment of cardiomyocyte content in biopsy. This new approach of mRNA expression assessment in microbiopsy samples was used to characterize calcium channel gene expression changes in cardiac allografts with and without diastolic dysfunction.

PCR Cloning of Target and Competitor Fragments

Isolated mRNA from normal left ventricular myocardium was reverse transcribed with the Superscript Preamplification System (Gibco). Polymerase chain reactions (PCRs) were performed in a PCR buffer of 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 1.4 mmol/L MgCl\(_2\), with 10 pmol/L of PCR primers and 2.5 U of Taq-polymerase in a final volume of 50 μL. All PCR programs consisted of 40 cycles at 94°C, annealing temperature (see below), and 72°C (1 minute each); and a final cycle of 5 minutes at 72°C. Amplification products were subcloned into pUC18. Recombinant cDNA was propagated in B1 and B2 host vector systems under S\(_{1}\) containment conditions according to German law. All cDNAs were sequenced on both strands by the dideoxy chain termination method (Sequenase, USB).

\[ \text{\( \beta \)-Subunit} \]

The 411-bp fragment (complementary to 788 to 1198; Reference 3) was PCR amplified with primers RH9 \(5'\)-GGATCTCCATCCTGCTGTA-3' and RH10 \(5'\)-GCTCGACGCTCCTCAATTTGCC-3' (annealing temperature, 60°C). Competitive construction was as depicted in Figure 1: PCR with RH9 and the internal linker primer RH11 \(5'\)-CATCCTCCAATTGCTCGAAGATTCGC-3' (matching a 19-bp sequence 262 bp upstream of the 3’-end of the 411-bp fragment) results in a 158-bp fragment. Thirteen bases of the 5’-end of this linker primer are identical to the 3’-strand end of RH10. PCR amplification of this 158-bp fragment with RH9 plus RH10 generates the 167-bp competitor fragment after PCR with RH9 and RH10, with identical primer binding sites to the 411-bp fragment but a deletion of 244 bp.

**\( \alpha_\text{c} \)-Subunit**

The 286-bp target fragment was amplified by PCR with primers RH1 \(5'\)-GCTCGACGCTCCTCAATTTGCC-3' and RH2 \(5'\)-GGACAGCAGATAGAAGCTGGGCTCGGACTCTGG-3' (complementary to 4006 to 4291; Reference 1) (annealing temperature, 60°C). The 226-bp \( \alpha_\text{c} \)-subunit competitor was constructed as described for the \( \beta \)-subunit competitor with the internal linker primer RH3 \(5'\)-ATGTAGAAGCTGGGCTCGGACTCTGG-3' (binding to a complementary sequence 76 bp upstream of the 3’-end of the 286-bp fragment) (Figure 2A).
**α/δ-Subunit**
For construction, see Reference 2 (Figure 2C).

**Calsequestrin**
A 190-bp fragment of coding sequence for calsequestrin (corresponding to 989 to 1179; Reference 26) was amplified with primers RH4 5'-GAGTTCCTGGAAATCCTGAA-3' and RH5 5'-ATCTCCATCCAGACACTGTC-3' (annealing temperature, 45°C) (Figure 3).

**Determination of mRNA Expression by Competitive PCR**
Biomagnetic separation (Dynal mRNA isolation kit) was used for mRNA isolation from single microbiopsy samples. Biopsy samples were homogenized on ice in a glass homogenizer in 40.2 μL of extraction buffer: guanidinium isothiocyanate 4 mol/L; Tris-HCl 0.1 mol/L (pH 8); DTT 1%; N-lauroylsarcosine 0.5%; and β-mercaptoethanol 3%. After addition of 79.8 μL of binding buffer (Tris-HCl 100 mmol/L, pH 8; LiCl 400 mmol/L; EDTA 20 mmol/L), the homogenate was sheared by drawing it into a hypodermic syringe fitted with a 23-gauge needle. After complementary binding of the poly(A) to the oligo(dT) Dynabeads, mRNA was separated magnetically. mRNA was eluted in EDTA 2 mmol/L (pH 8) at 65°C. Isolated mRNA was reverse transcribed into first-strand cDNA with the Gibco preamplification kit.

Competitive PCR analysis was set up in 4 separate reactions, each of which contained 4.5 μL of first-strand cDNA together with increasing amounts of the competitor fragments (see Figure 3) generated by cutting the subcloned competitor fragments out of the cloning vector, gel electrophoresis separation, electroelution, quantitation, and serial dilution. Competitive PCR protocols were identical to the PCR protocols
described above. PCR products were separated on a 6% polyacrylamide gel. PCR bands were visualized by ethidium bromide staining and analyzed by videodensitometry. Specific mRNA copy numbers per biopsy were calculated with correction factors of 3.2 (α1), 2.9 (α2), and 6.0 (β). Correction factors were computed from the ratio of target to competitor length multiplied by a factor of 2.5, accounting for the 40% (antipain, leupeptin, pepstatin, and benzamidine) and centrifuged for 35 minutes at 100 000 g. Protein concentrations in the resuspended pellets were determined by the bicinchoninic acid method.

Membrane proteins were separated on 7.5% SDS-polyacrylamide gels and either stained with Coomassie blue or transferred to nitrocellulose. The nitrocellulose membrane was blocked with Tris-HCl 10 mmol/L (pH 8.0) and NaCl 150 mmol/L containing 0.5% Tween and 3% bovine serum albumin and probed with the anti-βmorma antibody and an anti-rabbit IgG peroxidase-conjugated antibody. The anti-βmorma antibody was raised against the bacterially expressed 21-kDa core region common to all β-subunits. Detection was performed with the enhanced chemoluminescence system (Amersham).

Statistical Analysis
Results are presented as mean±SEM. For analysis of differences of mRNA expression at various left ventricular end-diastolic pressures (LVEDPs), the 2-tailed Student t test for unpaired data was used. The relation between β-subunit expression and LVEDP was analyzed by calculation of the Pearson product moment correlation coefficient ($r^2$). Correlation coefficients were calculated for linear or exponential fits; best fit was accepted. Probability values of $P<0.05$ were considered to be statistically significant.

Results
Patient Characteristics
The Table summarizes the hemodynamic data of all 72 cardiac allografts examined. In the LVEDP subgroups, LVEDP was not related to allograft age, number of rejections, or hypertension, all of which are factors described in the literature as being linked with diastolic dysfunction after transplantation. Furthermore, relevant macroscopic coronary stenosis was absent in all coronary angiograms. Ventriculography revealed normal left ventricular function in 68 patients (EF 74±1%; EDV 123±4 mL); 4 patients demonstrated reduced EF of 44% to 54%, 2 of whom had enlarged left ventricles (EDV 205 and 307 mL, respectively). In all cardiac allografts, left ventricular function was homogeneous, and systolic pressure rise in the isovolumic systolic phase remained well preserved even at high LVEDP (dP/dt 1691±130 mm Hg/s). Elevated LVEDP was related linearly ($r^2=0.86$) to impaired left ventricular relaxation as determined by the time constant $\tau$ in those patients in whom β-subunit expression was determined and who did not receive diltiazem (n=18/40). Therefore, the increase in LVEDP was considered to result from diastolic dysfunction in these patients.

Calcium Channel Subunit and Calsequestrin mRNA Expression in the Interventricular Septum and the Left Ventricle
Northern blots of septal or left ventricular normal human heart mRNA demonstrated equivalent expression of the calcium channel subunits and of calsequestrin (Figure 4), with minor variations between various septal and left ventricular samples (n=3; αc-subunit, 19±5%; αd-β-subunit, 18±8%; β-subunit, 15±7% [mean±SEM]). Therefore, septal microbiopsy samples were used to study calcium channel gene expression. αc-Subunit–specific antisense cRNA probes hybridized to transcripts of ~8 kb, and αd-β-specific cRNA probes detected 8-kb transcripts. β-subunit–specific antisense cRNA probes detected signals of 5.6 kb in normal allografts and in a failing cardiac allograft. This hybridization signal is larger than the 1.9- and 3.4-kb transcripts described previously. Antisense human calsequestrin cRNA probes labeled 2.6-kb transcripts (n=3; 9±3%; mean±SEM between septal and left ventricular myocardium) which corresponds to transcript sizes in other cardiac tissues.

Competitive RT-PCR of Cardiac Calcium Channel Subunits
The target fragments of the αc (286 bp), αd-β (293 bp), and β-subunits (411 bp) were amplified from a normal human heart cDNA library. The fragments were identical in sequence to the published sequences. The αc (226 bp), αd-β (251 bp), and β-subunit (167 bp) competitors were deletion mutants with identical primer binding sites. These target/competitor fragment pairs demonstrated only minor (αc-subunit; Figure 2A) or no heteroduplex formation (αd-β and β-subunits; Figure 2B and 2C), which may result from hybrid formation of primers or target/competitor molecules and may hamper analysis. mRNA levels of each subunit were normalized to calsequestrin mRNA in each biopsy sample (Figure 3).
The influence of acute allograft rejection on 6-subunit mRNA levels was characterized in 4 consecutive microscopy samples of one patient with class II acute rejection. For treatment, this patient received high-dose prednisone. Successive biopsy samples showed regression of acute rejection from class II to Ib.21 Calsequestrin mRNA levels did not change significantly, whereas 6-subunit expression was reduced in the first biopsy sample (Figure 6). Therefore, in our study, only biopsy samples with pathohistological gradings less than or equal to class Ib were examined.

**β-Subunit mRNA and Protein Levels in Diastolically Failing Cardiac Allograft**

β-Subunit antisense cRNA probes hybridized to 5.6-kb transcripts in equivalent quantities in septal and left ventricular tissue of normal (Figure 7; lanes 1 and 2) and explanted diastolically failing cardiac allografts (Figure 7; lanes 3 and 4). Compared with normal heart, β-subunit mRNA was reduced by 72% in the interventricular septum (Figure 7; lane 1 versus lane 3) and by 76% in the left ventricular free wall (Figure 7; lane 2 versus lane 4). This demonstrates that β-subunit mRNA levels in the left ventricle and the interventricular septum change in a similar manner.

Although the Coomassie stain of the SDS-PAGE showed identical protein patterns in a normal and a diastolically failing heart (Figure 8a), the β-common-antibody detected demarcation of the 75-kDa β-subunit protein in left ventricle of the failing cardiac allograft (Figure 8b).
Discussion

Previous studies\textsuperscript{12–15} on calcium channel expression in heart failure were confined to explanted myocardium from either normal or terminally failing human hearts and provided no information on the dynamic process of gene expression. Therefore, we characterized mRNA levels in microbiopsy samples from the interventricular septum of orthotopic cardiac allografts with and without diastolic dysfunction. Heart transplant recipients were chosen as our study collective because they represent a more homogeneous subgroup of patients who may develop diastolic dysfunction after transplantation but have preserved systolic function in general.\textsuperscript{16,17} Despite well-preserved systolic function, \textasciitilde50\% of the patients had an increase of LVEDP up to 28 mm Hg (Table) associated with clinical signs of heart failure (New York Heart Association class II). In those patients who did not receive diltiazem, the increase of LVEDP correlated ($r^2=0.86$) with impaired diastolic relaxation. Hence, in agreement with previous reports,\textsuperscript{16,17} we assumed that diastolic dysfunction was the predominant reason for heart failure. Other factors suggested to result in cardiac allograft diastolic dysfunction, such as arterial hypertension, the number of episodes of allograft rejection, age, or hemodynamically relevant macroscopic coronary artery stenosis,\textsuperscript{17} were not related to the increase in LVEDP (Table). This may be due to the small number of patients (n=72) or incomplete consideration of other parameters, such as hypoxia\textsuperscript{12} induced by allograft vasculopathy prevailing in the microcirculation, which is not analyzed by coronary angiograms.\textsuperscript{13} Also, other factors that may result in diastolic failure, such as hypertrophy or wall stress,\textsuperscript{34} were not considered.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4}
\caption{Left to right, Northern blotting demonstrates equivalent mRNA expression for $\alpha_1$, $\alpha_2/\delta$, and $\beta$-subunits and for cardiac calsequestrin in interventricular septum and left ventricular free wall. Exposure times were 7 days ($\alpha_1$ and $\alpha_2/\delta$), 60 hours ($\beta$), and 42 hours (cardiac calsequestrin). LV indicates left ventricle.}
\end{figure}
In our study, microbiopsy samples were obtained from the interventricular septum by right ventricular access. These specimens can be used to study gene expression in the left ventricle under the condition of equivalent gene expression in all parts of the left ventricle. In our Northern blot experiments, calcium channel subunit and calsequestrin mRNA levels were equivalent in the septum and the left ventricular free wall (Figure 4), in accordance with pharmacological studies that demonstrated equal distribution of dihydropyridine receptors in human heart.35 Furthermore, in an explanted failing cardiac allograft, β-subunit mRNA levels were reduced similarly in the septum and left ventricle. In our patients, left ventricular angiograms of cardiac allografts provided no evidence for regional myocardial dysfunction but showed homogeneously contracting ventricles even when LVEDP was severely increased. This lack of regional dysfunction, together with the apparently homogeneous changes in gene expression of the β-subunit (Figures 7 and 8), leads to the conclusion that in the present study, microbiopsy samples from the interventricular septum are representative of gene expression in the ventricle. However, minor differences of regional gene expression cannot be excluded.

Quantitative assessment of mRNA levels of cardiac calcium channel subunits in these microbiopsy samples was
performed by competitive RT-PCR with competitor fragments shortened by internal deletions but with identical primer binding sites. Thus, primer annealing kinetics were identical, and amplification kinetics were related. Quantitative analysis of mRNA levels in microisolation samples may be hampered by methodological limitations such as differences in amplification kinetics and reverse transcription efficiency. In a Northern blot with mRNA isolated from a diastolically failing cardiac allograft, β-subunit mRNA level reduction (Figure 7) was equivalent to the competitive β-subunit RT-PCR results, proving the validity of our competitive RT-PCR. In cardiac allografts with normal LVEDP, the numbers of auxiliary calcium channel subunits mRNA molecules corresponded, which further supports the accuracy of mRNA assessment by this method. The αI-subunit copy number was somewhat higher in all microisolation samples examined, which may reflect different primer annealing kinetics, different mRNA stability, or amplification of αII-subunits in cells other than cardiomyocytes associated with other regulatory subunits.

In the Northern blot, the antisense-cRNA probe derived from the β-subunit RH9/RH10 amplification probe detected longer transcripts than described elsewhere. Because of the sequence identity of the hybridization probe to the cardiac β-subunit and the stringent hybridization conditions, this signal may represent β1α-subunit coding mRNA, as can be expected from genomic analysis. Furthermore, the size of the β-subunit protein detected by the β1α-subunit antibody is similar to β1β-subunits. In our immunoblot (Figure 8), this 75-kDa protein was detected in total cardiac protein, whereas the 80-kDa β-subunit was detected only in purified human cardiac dihydropyridine receptors. Furthermore, the β1α-subunit antibody had precipitated only 25% of the radioactively labeled cardiac dihydropyridine receptors. Therefore, although the β1α-subunit antibody may also detect cardiac β2-subunits, the findings suggest that the β1α-subunit is the major β-subunit expressed in human heart.

Because of the important functional role of the β-subunit, the 4- to 5-fold reduced β-subunit expression that we demonstrated in this study can be expected to result in changes of the calcium current. Coexpression experiments in Xenopus oocytes have demonstrated a shift of calcium channel inactivation from maximal inactivation rates at a molar cRNA ratio of β-subunits to αI-subunits of >3:1 to slower rates at ratios <3:1. Furthermore, changes in neurohumoral modulation of the L-type calcium current are likely, because the β-subunit is involved in β-adrenergic signal transduction. To characterize the pathophysiological relevance of the association of decreased β-subunit expression with diastolic heart failure, directed coexpression studies are necessary. However, in the present study, we were able to examine mRNA levels in endomyocardial biopsy samples using competitive RT-PCR. This new approach enables the characterization of gene expression changes at early stages of heart disease.

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