Cardiac L-type Calcium Channel β-Subunits Expressed in Human Heart Have Differential Effects on Single Channel Characteristics*

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L-Type calcium channels are multiprotein complexes composed of pore-forming (Ca1.2) and modulatory auxiliary αδ- and β-subunits. We demonstrate expression of two different isoforms for the β2-subunit (β2a, β2b) and the β3-subunit (β3a, β3trunc) in human non-failing and failing ischemic myocardium. Quantitatively, in the left ventricle expression of β3a transcripts prevails in the order of > β3a > β3trunc. The expressed cardiac full-length β3-subunit is identical to the β3a-isoform, and β3trunc results from deletion of exon 6 (20 nn) entailing a reading frameshift and translation stop at nucleotide position 495. In failing ischemic myocardium β3trunc expression increases whereas overall β3 expression remains unchanged. Heterologous coexpression studies demonstrated that β2a induced larger currents through rabbit and human cardiac Ca1.2 pore subunits than β2b isoforms. All β-subunits increased channel availability at single channel level, but β2b exerted an additional, marked stimulation of rapid gating (open and closed times, first latency), leading to higher peak current values. We conclude that cardiac β-subunit isoforms differentially modulate calcium inward currents because of regulatory effects within the channel protein complex. Moreover, differences in the various β-subunit gene products present in human heart might account for altered single channel behavior found in human heart failure.

In human myocardium expression of three different β-subunit genes (β1-β3) was demonstrated at the mRNA and the protein level (3, 10). In human non-failing (NF) left ventricular (LV) mRNA our RT-PCR experiments with degenerated primers complementary to coding sequences with high similarity between β2- and β3-genes generated three amplification products of 650, 503, and 483 bp. The 503- and 483-bp amplification products are sequence-identical to β2 and β3 coding sequences (β2: GenBank™ accession number AF423189, nn 97–746; β3: GenBank™ accession number X76555, nn 135–637). The 483-bp amplification product contained a deletion of 20 nucleotides matching exon 6 of the β3-gene (11). At least two different isoforms of the human β3-subunit gene are expressed in the human heart (β3a, β3trunc) (12, 13) that differ with respect to their short amino termini. Our Northern blot experiments revealed transcripts for β3a, β3trunc, and β3-subunits in human heart tissue; therefore, we assessed quantitative expression of these β-subunits by real-time PCR, which demonstrated substantially different expression levels with β3b > β3a > β3trunc. To investigate the physiological effects of these β-subunits, we first cloned both the full-length coding sequence of the β3-subunit (β3a) and the isoform containing the deletion of exon 6 (20 nn) (β3trunc) from human heart. Deletion of exon 6 results in fibrillation or hypertrophic obstructive cardiomyopathy Ca1.2-subunit expression is altered, but in human diastolic or end stage heart failure most studies have demonstrated no change (see Refs. 1–4). Our studies, however, revealed alterations of auxiliary β-subunit expression, which is reduced in diastolic heart failure of cardiac allografts (3) and possibly in end stage heart failure (5). Down-regulation of β-subunit mRNA expression is a phenomenon not restricted to cardiac tissue, because similar observations were made in pancreatic islets of diabetic rats (6). Although an altered stoichiometry of β-subunits relative to the Ca1.2-subunits theoretically suggests variation in calcium inward current, we and others have detected no change in whole-cell calcium inward currents in cardiomyocytes isolated from human failing heart. Instead, calcium channel inward current was even paradoxically increased at the single channel level and could not be further augmented by cAMP-dependent phosphorylation (5). Because products from all four β-subunit genes modulate the ion conducting pore in a specific manner (7), we decided to study cardiac β-subunit gene expression in the human heart as a plausible reason for increased single calcium channel activity in heart failure.

1 The abbreviations used are: NF, non-failing; LV, left ventricular; RT, reverse transcriptase; ICM, ischemic cardiomyopathy; RA, right atrium; RV, right ventricle; CHO, Chinese hamster ovary; HER, human embryonic kidney; GFP, green fluorescent protein; ANOVA, analysis of variance; nn, nucleotide.
truncation of the protein, and, interestingly, β_trunc is up-regulated in heart failure. Because of the high homology of the rabbit β_csv-subunit to the human β_csv we used this subunit together with the two cloned human β_csv-subunits isoforms to study the functional impact of these β_csv-subunits onto the calcium inward current at the single channel level. Electrophysiological studies were designed to answer the following questions. 1) Do human cardiac β_csv-subunits functionally coexpress with pore subunits from rabbit heart (Ca_1.2a, rabbit smooth muscle (Ca_1.2b), and human heart (Ca_1.2)? Is there a difference in magnitude and extent of their modulation when coexpressed with a β_csv-subunit? 2) Does alternative splicing of the β_csv-subunits in heart failure explain the increase in single channel activity? These questions were addressed by transient coexpression in cell lines stably expressing Ca_1.2 isoforms.

**EXPERIMENTAL PROCEDURES**

**Tissue Specimens**—After local ethics committee approval, human myocardium specimens were obtained from explanted NF hearts not transplanted for technical reasons or from patients receiving orthotopic heart transplantation because of end-stage heart failure due to ischemic cardiomyopathy (ICM). All specimens were frozen directly after explantation.

**PCR-based Analysis of β_subunit Gene Expression in Human Myocardium**—The experiment is not depicted in this paper. Degenerated oligonucleotide primers complementary to coding regions of high similarity between human β_csv- and β_csv-subunits were obtained from MWG Biotech (Freising, Germany) (sense, 5'-CTGGAGAGACCGCAGAGCGAGAGGCG-3' (nn 356-376, X76555) and antisense primer 2, 5'-TGTTGTATGGTGTGAGGACTAAGTC-3' (nn 865-885, GenBank™ accession number X76555) with 0.1 µg of reverse-transcribed mRNA isolated from NF human LV myocardium in 1× PCR-Mix containing 1.5 mmol/liter MgCl₂, (pmol/µl) primers 0.2, dNTP 200, and 2.5 units of Taq polymerase (MBIfermentas, St. Leon Rot, Germany). Amplification products were separated on 0.8% agarose gels and DNA bands were excised, extracted by QuikSpin columns (Qiagen), subcloned into pTAdvantage (Clontech), amplified, and sequenced.

Ratio of β_csv-β_csvsubunit expression in human specimens was investigated by RT-PCR (40 cycles at 94, 60, and 72 °C, each 30 s) using sense primer 1, 5'-GCCGCTAGTGAAGAGGGCGCG-3' (nn 356-376, X76555) and antisense primer 2, 5'-GGTTGTATGGTGTGAGGACTAAGTC-3' (nn 865-885, GenBank™ accession number X76555) with 0.1 µg of reverse-transcribed mRNA isolated from NF- and ICM-LV (each n = 7). Amplification products were electrophoresed on 5% PAGE (see Fig. 1C), and densitometric analysis of amplified bands was performed by Quantity One (Bio-Rad). The experiment is not depicted in this paper. Degenerated oligonucleotide primers complementary to coding regions of high similarity between human β_csv- and β_csv-subunits were obtained from MWG Biotech (Freising, Germany) (sense, 5'-CTGGAGAGACCGCAGAGCGAGAGGCG-3' (nn 356-376, X76555) and antisense primer 2, 5'-TGTTGTATGGTGTGAGGACTAAGTC-3' (nn 865-885, GenBank™ accession number X76555) with 0.1 µg of reverse-transcribed mRNA isolated from NF human LV myocardium in 1× PCR-Mix containing 1.5 mmol/liter MgCl₂, (pmol/µl) primers 0.2, dNTP 200, and 2.5 units of Taq polymerase (MBIfermentas, St. Leon Rot, Germany). Amplification products were separated on 0.8% agarose gels and DNA bands were excised, extracted by QuikSpin columns (Qiagen), subcloned into pTAdvantage (Clontech), amplified, and sequenced.

Cloning of β_csv—Full-length β_csv-subunit sequence was cloned using two pairs of sequence-specific primers derived from X76555. 1st pair, 5'-GTCGATAGCTTGTAGTACCCCTTC-3' and 5'-GTTCCGACGCTGCGTGCTTC-3' (nn 437-454 and 1508-1521) and 2nd pair, 5'-GGTTGAGGACTAAGTC-3' (nn 39-84). Amplification products were electrophoresed on 5% PAGE (see Fig. 1C) and excised, extracted by QuikSpin columns (Qiagen), subcloned into pTAdvantage (Clontech), amplified, and sequenced.

**Cell Culture and Transfection**—Cell culture and transient transfection were done as described (15, 16). In brief, Chinese hamster ovary (CHO) cells were stably transfected with the Ca_1.2a-subunit cloned from rabbit heart (17) or with the Ca_1.2b-subunit from rabbit lung (18). Human embryonic kidney cells (HEK 293) were stably transfected with cDNA encoding the human cardiac Ca_1.2-subunit (NM_007119) (19). For experiments with pore subunits alone, cells were seeded in polystyrene Petri dishes (9.6 cm²; Falcon, Heidelberg, Germany) at densities between 10⁴ and 2 × 10⁵ cells/cm² and used within 48–96 h after plating.

For eukaryotic expression cloned human β_csv, β_csv, and rabbit β_csv subunits were used. The following subunits were coexpressed in CHO or HEK 293 cells were transiently cotransfected with the cDNA encoding the human cardiac Ca_1.2-subunit (NM_007119) (19). For experiments with pore subunits alone, cells were seeded in polystyrene Petri dishes (9.6 cm²; Falcon, Heidelberg, Germany) at a density between 10⁴ and 10⁵ cells/cm² and used within 48–96 h after plating.

Northern Blot Analysis—For the β_csv-subunit 10 µg of mRNA isolated with Trizol (Invitrogen) and poly(A) tract kit (Promega) from human right atrium (RA), right ventricle (RV), and LV obtained from NF/ICM hearts were used for northern blot analysis. mRNA isolation and Northern blot experiments were performed essentially as described before (11). Northern blots were hybridized at high stringency (18 h at 42 °C in 5× SSC, 1× PE (50 mm Tris-HCl (pH 7.5), 0.1% sodium diphosphate, 1% sodium dodecyl sulfate, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 5 mm EDTA), 50% formamide, with 2 × 10⁶ cpm/ml of a 32P-labeled antisense RNA probe derived from the β_csv-subunit-specific 503-bp amplification product (described above) subcloned into pSP72 containing phage T7 promoter activity, 1× 10⁶ cpm/ml). Northern blots were washed for 5 min at room temperature in 2× SSC, 0.1% SDS, 10 min in 0.2× SSC, 0.1% SDS, and then at 50 °C in 0.2× SSC, 0.1% SDS (NF, 15 min; ICM, 15 + 10 min). Autoradiography exposure was 96–108 h at −80 °C on Eastman Kodak Co. BioMax MS-1 (Amersham Biosciences) for NF/ICM Northern blots. For the β_csv-subunit 2 µg of NF LV/RV, ICM LV/RV mRNA were electrophoresed, blotted, and hybridized with isoform-specific 32P-labeled β_csv and β_csv (nn 78955–78947 and 158721–159133 respectively; GenBank™ accession number AL390783) and C-terminal-specific β_csv (nn 1374–2327, GenBank™ accession number U95019). Antisense RNA probes were subcloned into pGEM-3Z (Promega) (β_csv), pC 2.1-TOPO (Invitrogen) (β_csv), and pTAvantage (Clontech) (β_csv). The experiment is not depicted in this paper. Degenerated oligonucleotide primers complementary to coding regions of high similarity between human β_csv- and β_csv-subunits were obtained from MWG Biotech (Freising, Germany) (sense, 5'-CTGGAGAGACCGCAGAGCGAGAGGCG-3' (nn 356-376, X76555) and antisense primer 2, 5'-TGTTGTATGGTGTGAGGACTAAGTC-3' (nn 865-885, GenBank™ accession number X76555) with 0.1 µg of reverse-transcribed mRNA isolated from NF human LV myocardium in 1× PCR-Mix containing 1.5 mmol/liter MgCl₂, (pmol/µl) primers 0.2, dNTP 200, and 2.5 units of Taq polymerase (MBIfermentas, St. Leon Rot, Germany). Amplification products were separated on 0.8% agarose gels and DNA bands were excised, extracted by QuikSpin columns (Qiagen), subcloned into pTAdvantage (Clontech), amplified, and sequenced.

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EGTA 10, HEPES 5 (pH 7.4). Ba\(^{2+}\) currents were elicited at 0.2 Hz by depolarizing voltage steps from a holding potential of \(-80\) mV to various test potentials as indicated. Currents were sampled at 10 kHz and filtered \((-3\) dB) at 2kHz (List EPC-9; HEKA, Lambrecht, Germany). Leak and capacitive currents were subtracted by using a P/N pulse protocol. Peak currents were determined using the average of a 5-ms time window. Time-dependent inactivation was analyzed as \(I_{100}\), the current remaining at the end of a 100-ms test pulse, relative to peak current. The software PULSE/PULSE-FIT (version 9.12; HEKA) was used for data acquisition and analysis.

Single channel measurements and analysis were done as reported (5). Cells were superfused with bath solution containing (mmol/liter) potassium glutamate 120, KCl 25, MgCl\(_2\) 2, HEPES 10, EGTA 2, CaCl\(_2\) (5). Cells were superfused with bath solution containing (mmol/liter) BaCl\(_2\) 110, HEPES 10 (pH 7.4 with tetraethylammonium hydroxide). Single calcium channels were recorded in the cell-attached configuration (depolarizing test pulses of 150-ms duration at 1.67 Hz, holding potential \(-100\) mV). An Axopatch 1D amplifier and PClamp 5.5 or 6.0 software (both Axon Instruments, Foster City, CA) were used for pulse generation, data acquisition (10 kHz), and filtering (2 kHz, \(-3\) dB, 4-pole Bessel filter). Experiments were analyzed whenever the channel activity persisted for at least 72 \(\times\) (120 sweeps, using 180 sweeps in most cases). Linear leak and capacity currents were subtracted digitally. Openings and closures were identified by the half-height criterion. The availability (fraction of sweeps containing at least one channel opening), the open probability \((p_{\text{open}}; \text{defined as the relative occupancy of the open state during active sweeps})\), and the peak ensemble average current \((I_{\text{peak}})\) were obtained visually) were analyzed from single channel and multichannel patches. In the latter case, they were corrected for \(n\), the number of channels in the patch. \(n\) was defined as the maximum current amplitude observed, divided by the unitary current. Peak current was corrected by division through \(n\). The availability was corrected by the square root method: \((1 - \text{availability}_{\text{corrected}})\) is the \(n\)th root of \((1 - \text{availability}_{\text{uncorrected}})\). The corrected \(p_{\text{open}}\) was calculated on the basis of the corrected number of active sweeps, i.e. total open time divided by \((n \times \text{availability}_{\text{corrected}} \times \text{number of test pulses} \times \text{pulse length})\). Closed time and first-latency analyses were carried out in patches where \(n = 1\). Time constants of open and closed time histograms were obtained by maximum-likelihood estimation (PStat software; Union City, CA and Axon Instruments).

Data Analysis—Effects of the \(\beta\)-subunits on single channel parameters were statistically examined by one-way ANOVA over the whole series \((i.e.\text{untransfected controls of a given pore subunit and cells cotransfected with }\alpha_{\text{delta}}, pGFP, \text{and pcDNA3.1 or a given }\beta\text{-subunit subcloned into pcDNA3.1})\). Significant ANOVAs were followed by post-tests against pcDNA3.1 and among \(\beta\)-subunits, applying Bonferroni correction for multiple comparisons. \(p < 0.05\) was considered significant. All values are given as mean \(\pm\) S.E.

RESULTS

Specific antisense \(\beta_2\)- and \(\beta_3\)-RNA probes hybridized to distinct transcripts in Northern blots with human LV NF and ICM mRNA. The \(\beta_2\)-specific probe hybridized to two major transcripts in RA, RV, and LV in both human NF and ICM. The sizes of the large transcripts vary with respect to disease state and myocardial localization, 3.1 kb in NF RA, 3.1 kb in NF RV, 3.5 kb in NF LV, 3.5 kb in ICM RA, 2.9 kb in ICM RV, and 3.5 kb in ICM LV whereas the smaller transcript size was 1.1 kb in all heart tissues examined but 0.8 kb in the ICM RV (Fig. 2A). Because no major alternative splicing within the coding sequence was observed with cloning of \(\beta_2\) by RT-PCR, variable transcript sizes must be because of sequence differences in either the 5’- or 3’-untranslated regions. Major transcripts detected by the \(\beta_2\)-specific probe were 9.5, 3.8, and 2.7 kb in NF/ICM LV/RV mRNA (Fig. 2A) whereas the \(\beta_3\)-specific probe hybridized almost exclusively to 9.5-kb transcripts in the tissues examined (Fig. 2A). Because \(\beta_2\) and \(\beta_3\) coding sequences differ only with respect to their short N-terminal sequences, isoform-specific antisense probes contained major parts of isoform-specific 5’-untranslated sequences. Therefore, we proved the identity of the transcripts detected using a \(\beta_2\text{common}\) probe directed against C-terminal coding sequence common to human \(\beta_2\) and \(\beta_3\). This probe marked transcripts of similar sizes and also another 11.2-kb transcript in NF LV that is barely detectable in ICM LV (Fig. 2A). The specific probe for the housekeeping gene cardiac calceuscin hybridized to major transcripts of 2.6 kb in NF and ICM LV mRNA as described above (3, 5). Generally, Northern blot intensity of all transcripts detected in RV tissues was less strong compared with LV tissues detected similar total mRNA loading reflecting left ventricular myocyte to matrix dominance.

Abundance of the \(\beta_2\)-, \(\beta_3\)-, and \(\beta_3\)-subunit mRNA expression was determined by real-time RT-PCR in NF LV mRNA \((n = 3)\) and ICM LV mRNA \((n = 5)\; mean\) echocardiographic ejection fraction of examined ICM hearts, 21 \(\pm\) 2.23% (S.D.)). Expression data in each specimen were normalized to the respective expression of the housekeeping gene cardiac calceuscin \((3, 21)\), which was also determined by real-time RT-PCR. Real-time RT-PCR for \(\beta_2\)/\(\beta_3\) was designed with isoform-specific N-terminal sense primers but common C-terminal antisense primer and fluorescent probe. Because we detected no significant differences of copy numbers among \(\beta_2\), \(\beta_3\), and \(\beta_3\) when comparing NF LV and ICM LV, data were pooled. In the specimens examined the \(\beta_2\) copy number was \(1.49 \times 10^5 \pm 6.18 \times 10^4\) which is 35 times the \(\beta_2\) copy number of \(4.11 \times 10^3 \pm 1.42 \times 10^3\) \(p = 0.01\) (Fig. 2C). Real-time RT-PCR for the \(\beta_3\) detected \(4.83 \times 10^5 \pm 1.48 \times 10^5\) copies in the eight specimens examined, which was significantly differ-
ent from either $\beta_{2a}$ ($p = 0.02$) or $\beta_{2b}$ ($p = 0.03$) (Fig. 2C). PCR efficiencies of $\beta_{2a}$, $\beta_{2b}$, $\beta_3$, and cardiac cardiac calsequestrin were very close to 100%, and correlation coefficients were $>0.99$, respectively (see "Experimental Procedures").

Data were pooled because of non-significant differences between NF/ICM. Expression levels for $\beta_{2a}$, $\beta_{2b}$, and $\beta_3$ were normalized to the expression of cardiac calsequestrin hybridizes to 2.6-kb transcripts in LV from NF/ICM. C, real-time RT-PCR was performed with LV specimens from NF ($n = 3$) and ICM ($n = 5$). mRNA copy number was calculated on the basis of a 40% efficiency of reverse transcription, providing a solid basis for comparison.

The full-length $\beta_{2a}$-sequence cloned from human NF LV mRNA by RT-PCR consists of 1455 bp and encodes 484 amino acids, and this $\beta_3$ is identical to the $\beta_{2a}$-subunit isoform cloned from human thyroidoma (GenBank accession number X76555). Except for exon 6 deletion (20 nt), which results in a premature stop of translation at nucleotide position 495 no other splicing product of the $\beta_3$-gene was observed in human heart. A similar truncation was also observed in mouse brain tissue (23). Because truncated proteins may play a role in cardiomyopathy, as demonstrated for troponin I (24), we further investigated the (patho-)physiological relevance of $\beta_{3\text{trunc}}$ by performing RT-PCR experiments using primers 1 + 2 (Fig. 1B) in LV mRNA isolated from NF and ICM (Fig. 1C). In the specimens examined (LV NF/ICM, each $n = 7$; mean echocardiographic LV ejection fraction in ICM patients, 23.75 ± 2.75% (S.D.)) the ratio of $\beta_{2a}/\beta_{3\text{trunc}}$-subunit isoform expression changed from 83/17% in NF LV to 51/49% in ICM LV.

CHO cells stably expressing the different splice variants of the Ca$_{\alpha,1.2}$ L-type channel pore were transiently cotransfected with $\alpha_{\alpha,\delta}$-subunits together with either rabbit $\beta_{2a}$- or the cloned human $\beta_{3\text{trunc}}$- or $\beta_{3\text{trunc}}$-subunits (Fig. 3). The existing rabbit $\beta_{2a}$-clone was chosen for our coexpression experiments because of its high homology (96%) to the $\beta_{2a}$, which in the human heart is predominantly expressed when compared with human $\beta_{2a}$. Overall, cotransfection increased current density, shifted the current-voltage relationship toward more negative potentials, and increased the rate of inactivation, e.g. at the respective potential of peak current density ($I_{\text{100}}$ for non-cotransfected cells, 102 ± 3%; $\beta_{2a}$, 66 ± 9%; $\beta_{3\text{trunc}}$, 74 ± 4%; and $\beta_{3\text{trunc}}$, 68 ± 13%; $n$ as in Fig. 3), as reported (12, 25). Although the qualitative effects with cotransfection of rabbit $\beta_{2a}$ and human $\beta_3$-subunits were similar, the effects of $\beta_{3\text{trunc}}$-subunits, and of $\beta_{3\text{trunc}}$ in particular, were less pronounced.
multiple splice variants of human cardiac-type Cav1.2 (27, 28), we next examined the Cav1.2 pore-forming subunit usually expressed in human myocardium (19). We expressed cDNAs in HEK 293 cells for these studies, which enabled us to perform a more extended analysis, because of better seal stability. The inherent properties of pore subunits, as well as their differential modulation by rabbit β2a and human β3 isoforms (Table III), were comparable with those of the CHO system. Another common feature with the CHO cell experiments was recognized: β2a, but not the β3-subunits increased open probability and open time duration. Closed time duration appeared to be shortened by β2a mainly because of a change of its slow component. As expected, the first latency was shortened by β2a, coexpression. β3strunc had no effects on fast gating, and β2a took a numerically intermediate position regarding these parameters. The effect of β3strunc on availability and peak current fell visibly short of the effect of β2a in this expression system. Finally, we examined a relevant window of test potentials (0 to +20 mV) where voltage-dependent activation of gating parameters rises steeply. This reveals the biophysical nature of the differential modulation of the pore subunit by β2a versus β2a and β3strunc (Fig. 6). With β2a, the voltage dependence of open probability was shifted to the left. This was not as prominent with β2a and even less with β3strunc. Unfortunately, more positive potentials could not be examined because of bandwidth limitation (29). However, it becomes clear that the amount of voltage shift is larger with β2a. Considering availability, modulation by β2a and β2a again were quite similar (not shown).

A detailed comparison, and separation of possible effects of cotransfected αδ-subunits, was carried out using the single channel technique.

Typical traces from single channel experiments using the recombinant Ca1.2a pore-forming subunit are depicted in Fig. 4. Single channel activity was sparse in patches from non-cotransfected as well from cells cotransfected with αδ-subunits (plus pcDNA3.1 and pGFP) only. β2a and β3strunc increased the probability of finding openings within a test pulse (availability). Open probability within such active sweeps appeared moderately enhanced. In contrast, rabbit β2a cotransfection led to marked increases in both parameters and to a higher increase in the peak current of the ensemble average. These visual impressions were statistically confirmed (Table I). Analysis of fast gating parameters in one-channel patches revealed that the mean closed time is the parameter most prominently affected by rabbit β2a, whereas human β2a or β3strunc have no effect on closed times. Histogram analysis showed that the reduction in mean closed times is because of a shortening of the time constant of the slow component \( \tau_{\text{closed,slow}} \). Open time histograms revealed an increase in the time constant of the open state (Table I) but only in the case of rabbit β2a.

To examine whether the more marked modulation by rabbit β2a-subunits is restricted to the cardiac type Ca1.2a pore subunit, we examined the effect of β2a and β3a on the smooth muscle splice variant, Ca1.2b. As indicated in Fig. 5 and Table II, very similar results were found as with Ca1.2a. Again, both β-subunits increased availability similarly. Ensemble average currents were more markedly affected by β3a because of a significant additional effect on rapid gating and open probability. Similar to Ca1.2a, \( \tau_{\text{closed,slow}} \) was reduced, which may explain this effect. Together, these findings confirm a role of the β-subunit structure on pore properties. As with Ca1.2a, non-transfected cells and cells cotransfected only with pcDNA3.1, αδ-subunits, and GFP displayed a quite sparse pattern of activity of Ca1.2b (Fig. 5), similar to our previous observations (26).

To account for the functional differences found among the different αδ-subunit isoforms, we introduce the idea of a high-affinity fast component (largely \( \beta \)) and a low-affinity slow component (largely \( \gamma \)). As expected, the fast component (\( \beta \)) was present for all substitutions, but the slow component (\( \gamma \)) was selective for rabbit and human. A high-affinity fast component reveals a high affinity for the 

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**FIG. 4.** Comparison of single recombinant mono- and heteromultimeric L-type calcium channel complexes from CHO-OzH10 cells stably expressing rabbit cardiac Ca1.2a. Top row, pulse protocol (150-ms pulse length, holding potential −100 mV, test pulse +10 mV, applied every 600 ms). Middle, 20 consecutive single traces for each channel complex (transiently cotransfected with additional αδ-subunit and GFP cDNA vectors, except for non-cotransfected control). Bottom row, ensemble average current of all traces of each experiment (≥120 sweeps). Scale bars, 10 ms, 2 pA (unitary current traces), or 100 fA (ensemble average current).

**DISCUSSION**

In the present study, we describe expression of two β2-subunit isoforms (β2a, β2b) and of two β3-subunits (β3a, β3trunc) in the human heart. The β2a isoform-specific probe detected distinct transcripts of 9.5, 3.8 and 2.7 kb in left ventricular mRNA from human non-failing and failing ischemic cardiomyopathy whereas the β2a-specific probe hybridized to a major transcript of 9.5 kb in both specimens. Because of the short length of the isoform-specific N-terminal coding sequences, each antisense probe contained untranslated sequences to major parts that might anneal non-specifically to other non-coding sequences. To prove the specificity of the hybridization signals we therefore used a β3common antisense RNA probe complementary to the coding C-terminal sequence common to both β2a and β2b. This probe detected transcripts of similar sizes, thus, unspecific hybridization of the isoform-specific probes is unlikely. Our probes detected transcript sizes different from the sizes observed by Freise et al. (8) who demonstrated in human heart mRNA a major band of 3.5 kb and a faint band of 6.9 kb when using a radioactively labeled murine β2 145-bp cDNA probe. Differences in the transcript sizes detected may result either from experimental discrepancies or inherent properties of the various probes. However, in our Northern blots the detection of transcripts of similar sizes by two different antisense RNA probes that recognize diverse sequences of the same mRNA molecule was re-assuring. Our probes marked relatively large transcripts, although full-length coding sequences of the β2a and β2b are 1818 and 1821 bp, respectively. We can rule out genomic contamination of our mRNA preparations, because β2a and β2b real-time RT-PCR from the same mRNA preparations resulted in single amplification products despite amplification across boundaries of N-terminal and common C-terminal coding exons. Most likely, these long transcripts represent progenitor forms of the finally translated mRNA molecules reflecting the large dispersion of the β2 gene over chromosome 10.

In contrast to earlier studies that did not detect β3-subunit mRNA expression in human LV mRNA (30), we detected transcripts of 3.5 kb and smaller sizes in RA, RV, and LV from both
human non-failing and failing ischemic myocardium. Transcripts of 3.5 kb were also reported for other human tissues (30). Both the expression of \( \beta_3 \) transcripts in the whole heart and the finding of differential splicing of exon 6 suggest a physiological role of the \( \beta_3 \) in the human heart, which is also consistent with the expression of \( \beta_3 \) in the whole heart with an increased gene dose, should have affected both parameters with post-hoc, two-tailed t tests.

| Parameter | \( \beta_{2a} + \alpha \delta \) | \( \beta_{2a} + \alpha \beta \) | \( \beta_{2a} + \alpha \gamma \) | \( \beta_{2b} + \alpha \delta \) | \( \beta_{2b} + \alpha \beta \) | \( \beta_{2b} + \alpha \gamma \) | \( \beta_{3} + \alpha \beta \) | \( \beta_{3} + \alpha \gamma \) |
|-----------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| \( I_{\text{patch}} \) (fA) | 149 ± 63 | 36 ± 7 | 26 ± 5 | 70 ± 10 | 6 | 12 ± 1 | 9 |
| Availability (\%) | 56.6 ± 18.9 | 76.4 ± 6.5 | 68.1 ± 7.5 | 27.3 ± 9.3 | 6 | 42.6 ± 7.9 | 5 |
| \( p_{\text{open}} \) (\%) | 3.8 ± 1.6 | 1.8 ± 0.3 | 1.1 ± 0.2 | 0.59 ± 0.21 | 6 | 0.97 ± 0.17 | 9 |
| Open time (ms) | 0.37 ± 0.04 | 0.19 ± 0.02 | 0.25 ± 0.05 | 0.18 ± 0.03 | 6 | 0.26 ± 0.03 | 9 |
| Closed time (ms) | 7.7 ± 2.9 | 7.8 ± 1.0 | 11.6 ± 3.0 | 6 | 19.3 ± 3.9 | 6 | 11.5 ± 1.3 | 8 |
| First latency (ms) | 28.6 ± 7.6 | 24.9 ± 5.2 | 40.3 ± 5.9 | 6 | 43.8 ± 6.6 | 6 | 35.9 ± 3.9 | 8 |
| \( \tau_{\text{open}} \) (ms) | 0.42 ± 0.10 | 0.19 ± 0.03 | 0.21 ± 0.03 | 0.24 ± 0.02 | 2 | 0.20 ± 0.02 | 9 |
| \( \tau_{\text{closed, fast}} \) (ms) | 0.47 ± 0.23 | 1.09 ± 0.30 | 1.30 ± 0.20 | 4 | 1.20 ± 0.90 | 2 | 0.75 ± 0.13 | 8 |
| \( \tau_{\text{closed, slow}} \) (ms) | 12.8 ± 3.5 | 11.9 ± 0.8 | 17.4 ± 2.7 | 4 | 21.1 ± 1.5 | 2 | 23.1 ± 2.9 | 8 |
| Fraction \( \tau_{\text{closed, fast}} \) | 0.41 ± 0.15 | 0.48 ± 0.10 | 0.56 ± 0.10 | 6 | 0.54 ± 0.22 | 2 | 0.41 ± 0.07 | 8 |

\( ^* p < 0.05 \) amongst groups.
\( ^\# p < 0.05 \) vs. pcDNA3.1.
\( ^\% p < 0.05 \) vs. \( \beta_{2a} \).

As with previous gene expression studies in human myocardium (3, 5), expression levels of \( \beta_{2a} \), \( \beta_{3} \), and \( \beta_{3} \) were all normalized to cardiac calsequestrin expression in the same sample. This sarcoplasmic calcium-binding protein was chosen as "housekeeping gene," because different studies in human heart failure showed that its expression is consistently unchanged at the mRNA and the protein level when compared with non-failing myocardium (21). Furthermore, cardiac calsequestrin is almost exclusively expressed in the cardiomyocyte (31); thus, in human heart expression of the gene of interest can be related to the cardiomyocyte content in the specimen examined.

Cellular localization of the \( \beta_{2a} \) and \( \beta_{3} \) subunit isoforms cannot be delineated from our gene expression studies, and appropriate experiments might also be crippled by their low abundance of expression. For this reason, we addressed this question at the functional level by expressing the \( \beta_{2a} \) and \( \beta_{3} \)-subunit in cells that stably express the CaV1.2a, CaV1.2b, and human CaV1.2. The calcium currents obtained by coexpression with the \( \beta_{3} \) isoforms were compared with currents induced by rabbit \( \beta_{2a} \) because of its 96% homology to the human \( \beta_{2b} \). Indeed, the \( \beta_{2a} \) and to a lesser extent the \( \beta_{3} \) splice variant, elicited functional impact when coexpressed with the cardiac pore-forming subunit. Interestingly, the extent of modulation by \( \beta_{3} \) was substantially less than with \( \beta_{2a} \). This difference is not because of a species mismatch between the rabbit and human clones, because similar findings were obtained with both rabbit and human cardiac pore-forming subunits. We also tend to exclude confounding effects of a different dose of the subunit isoforms; all technical precautions (same expression vector, same amount of plasmid DNA) were appropriately implemented, and, more convincingly, the distinctive features of single channel modulation by \( \beta_{2a} \) and \( \beta_{3} \) help to rule out this concern. Although \( \beta_{2a} \) and \( \beta_{3} \) equivalently increased channel availability, only \( \beta_{2a} \) showed a large and statistically robust stimulation of the fast gating properties. A more pronounced influence of the same kind of modulation, as with an increased gene dose, should have affected both parameters in parallel. One may argue that our single channel analysis could be biased by presence of a multitude of channels in the patch, even in cases where no multiple simultaneous openings were detected. However, this would even sharpen the distinction between \( \beta_{2a} \) and \( \beta_{3} \), because we can exclude multichannel patches with greater certainty in the case of \( \beta_{2a} \). The probability that the absence of stacked openings indicates the
Single Channel Effects of Calcium Channel β-Subunits

TABLE II

Single channel properties of recombinant L-type calcium channel complexes from transiently cotransfected HEK 293 cells stably expressing human cardiac Ca\textsubscript{v}1.2

| Parameter | β\textsubscript{3a} + α\textsubscript{δ} | n | β\textsubscript{3a} + α\textsubscript{δ} | n | pcDNA3.1 + α\textsubscript{δ} | n | Ca\textsubscript{v}1.2b control | n |
|-----------|-------------------------------|---|-------------------------------|---|-------------------------------|---|-------------------------------|---|
| I\textsubscript{peak} (fA)\textsuperscript{r} | 113 ± 23\textsuperscript{abc} | 6 | 63 ± 1\textsuperscript{a} | 16 | 35 ± 6 | 17 | 16 ± 2 | 12 |
| Availability (%)\textsuperscript{r} | 66.5 ± 8.0\textsuperscript{b} | 6 | 68.8 ± 8\textsuperscript{b} | 10 | 26.7 ± 3.7 | 10 | 33.3 ± 5.3 | 12 |
| p\textsubscript{open} (%)\textsuperscript{r} | 4.1 ± 0.8\textsuperscript{b} | 6 | 1.6 ± 0.3 | 10 | 0.79 ± 0.12 | 10 | 0.97 ± 0.17 | 12 |
| Open time (ms) | 0.36 ± 0.06 | 3 | 0.30 ± 0.05 | 6 | 13.2 ± 1.3 | 9 | 16.9 ± 3.4 | 12 |
| Closed time (ms) | 8.5 ± 2.7 | 3 | 14.4 ± 2.6 | 6 | 53.6 ± 2.8 | 9 | 48.1 ± 2.5 | 12 |
| First latency (ms)\textsuperscript{p} | 27.5 ± 7.0\textsuperscript{a} | 3 | 32.1 ± 3.5\textsuperscript{b} | 6 | 53.6 ± 2.8 | 9 | 48.1 ± 2.5 | 12 |
| τ\textsubscript{open} (ms) | 0.38 ± 0.09 | 3 | 0.27 ± 0.06 | 9 | 0.30 ± 0.08 | 9 | 0.33 ± 0.05 | 12 |
| τ\textsubscript{closed, fast} (ms) | 0.62 ± 0.17 | 3 | 0.79 ± 0.28 | 5 | 0.93 ± 0.24 | 9 | 0.63 ± 0.14 | 9 |
| τ\textsubscript{closed, slow} (ms)\textsuperscript{p} | 13.1 ± 3.3\textsuperscript{a} | 3 | 16.6 ± 2.4 | 5 | 24.8 ± 1.9 | 9 | 21.7 ± 1.8 | 9 |
| Fraction τ\textsubscript{closed, fast} | 0.42 ± 0.04 | 3 | 0.29 ± 0.05 | 5 | 0.50 ± 0.04 | 9 | 0.39 ± 0.07 | 9 |

\textsuperscript{a} p < 0.05 amongst groups.
\textsuperscript{b} p < 0.05 vs. pcDNA3.1.
\textsuperscript{c} p < 0.05 vs. β\textsubscript{3a}.

TABLE III

Single channel properties of recombinant L-type calcium channel complexes from transiently cotransfected HEK 293 cells stably expressing rabbit smooth muscle type Ca\textsubscript{v}1.2b

| Parameter | β\textsubscript{3a} + α\textsubscript{δ} | n | β\textsubscript{3a} + α\textsubscript{δ} | n | pcDNA3.1 + α\textsubscript{δ} | n | Ca\textsubscript{v}1.2b control | n |
|-----------|-------------------------------|---|-------------------------------|---|-------------------------------|---|-------------------------------|---|
| I\textsubscript{peak} (fA)\textsuperscript{r} | 113 ± 23\textsuperscript{abc} | 12 | 63 ± 11\textsuperscript{a} | 16 | 35 ± 6 | 17 | 16 ± 2 | 12 |
| Availability (%)\textsuperscript{r} | 71.3 ± 6.0\textsuperscript{b} | 12 | 64.1 ± 6.0\textsuperscript{b} | 16 | 56.3 ± 6.1 | 17 | 40.1 ± 5.9 | 12 |
| p\textsubscript{open} (%)\textsuperscript{r} | 5.9 ± 1.5\textsuperscript{a} | 12 | 3.3 ± 0.5 | 16 | 1.9 ± 0.3 | 17 | 1.1 ± 0.2 | 12 |
| Open time (ms)\textsuperscript{p} | 0.34 ± 0.04\textsuperscript{a} | 12 | 0.26 ± 0.01 | 16 | 0.24 ± 0.02 | 17 | 0.28 ± 0.04 | 12 |
| Closed time (ms) | 5.0 ± 0.5 | 5 | 12.4 ± 8.1 | 7 | 12.7 ± 2.8 | 10 | 10.4 ± 1.7 | 10 |
| First latency (ms) | 21.0 ± 6.8\textsuperscript{a} | 5 | 32.6 ± 5.3 | 7 | 43.7 ± 6.0 | 10 | 47.9 ± 5.0 | 10 |
| τ\textsubscript{open} (ms) | 0.26 ± 0.02 | 12 | 0.22 ± 0.01 | 16 | 0.18 ± 0.03 | 15 | 0.22 ± 0.01 | 12 |
| τ\textsubscript{closed, fast} (ms) | 0.70 ± 0.13 | 5 | 0.50 ± 0.1 | 6 | 0.91 ± 0.21 | 10 | 0.55 ± 0.16 | 8 |
| τ\textsubscript{closed, slow} (ms) | 9.5 ± 2.1 | 5 | 17.7 ± 5.2 | 6 | 20.9 ± 5.5 | 10 | 15.7 ± 1.4 | 8 |
| Fraction τ\textsubscript{closed, fast} | 0.50 ± 0.05\textsuperscript{a} | 5 | 0.26 ± 0.06 | 6 | 0.40 ± 0.06 | 10 | 0.34 ± 0.07 | 8 |

\textsuperscript{a} p < 0.05 amongst groups.
\textsuperscript{b} p < 0.05 vs. pcDNA3.1.
\textsuperscript{c} p < 0.05 vs. β\textsubscript{3a}.

FIG. 6. Analysis of voltage-dependent activity of the different recombinant cardiac mono- and heteromultimeric L-type calcium channel complexes from HEK 293 cells stably expressing human cardiac Ca\textsubscript{v}1.2. Fast gating parameter p\textsubscript{open}, (fraction of open time duration within active traces) is plotted versus test potential. Coexpression of β\textsubscript{3a}-subunit (■) had significant effects at more positive voltages, compared with co-transfected (●) and pcDNA3.1-transfected (○) channels. Coexpression of β\textsubscript{3b} (▲) and β\textsubscript{3trunc} (▼)-subunits had smaller, or no such effects, respectively.

The observed functional consequence of differential splicing of β\textsubscript{3a}-subunits cannot explain the increase in channel activity as observed in human heart failure (5). β\textsubscript{3trunc}-subunits, which appear to modulate the pore in a qualitatively similar manner as β\textsubscript{3a}, have a less pronounced influence in almost every respect. Although channel activity is typically doubled by β\textsubscript{3trunc}, this effect does not reach significance when corrected for the multiple comparisons we were obliged to perform. Viewed in isolation, even the β\textsubscript{3trunc} isofom is able to modulate the cardiac pore subunits, albeit in a more subtle manner. This is not unexpected, because N-terminal interaction sequences remain despite the premature translational stop, which results in loss of the principal β-subunit interaction domain and, furthermore, of potential phosphorylation sites for protein kinase C and the casein kinase II (30). Also, such structural features may alter interaction with G-proteins (35, 36). β\textsubscript{3trunc} expression may actually compete with other isoforms and thus reduce single channel activity in native heteromultimeric complexes, but channels behaving in such a manner were not detected in native failing human myocytes (5, 29, 37).

In summary, β-subunit gene products modulate cardiac calcium channel pores in an isoform- and splice variant-specific manner. Therefore, it is crucial to quantify not only the abso-

For closed time and latency analysis, only experiments containing just one detected open level were used for calculation. The other values were corrected for the number of detected current open levels indicating more than one single channel. One-way ANOVA was computed for all parameters with post-hoc, two-tailed t tests.

"...comparisons amongst groups."

"...amongst groups."
lute amount of mRNA or protein of these subunits under pathological conditions but rather the relative contribution of the subtypes actually expressed. Future studies should dissect such β-subunit alterations. These could serve as a molecular basis of altered single channel behavior in heart failure, which in turn may critically compromise excitation-contraction coupling.

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Cardiac L-type Calcium Channel β-Subunits Expressed in Human Heart Have Differential Effects on Single Channel Characteristics

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