Enhanced Expression of L-type Ca\textsubscript{v}1.3 Calcium Channels in Murine Embryonic Hearts from Ca\textsubscript{a}1.2-deficient Mice*

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Voltage-gated calcium (Ca\textsuperscript{2+}) channels play a key role in the control of heart contraction and are essential for normal heart development. The Ca\textsubscript{a}1.2 L-type calcium channel is the predominant isoform in cardiomyocytes and is essential for excitation-contraction coupling. Although the inactivation of the Ca\textsubscript{a}1.2 gene caused embryonic lethality before embryonic day E14.5, hearts were contracting before E14 depending on a dihydropyridine-sensitive calcium influx. We analyzed the consequences of the deletion of the Ca\textsubscript{a}1.2 channel on the expression level of other voltage-gated calcium channels in the embryonic mouse heart and isolated cardiomyocytes. A strong compensatory up-regulation of the Ca\textsubscript{a}1.3 calcium channel was observed on the mRNA as well as on the protein level. Reverse transcriptase PCR indicated that the recently identified new Ca\textsubscript{a}1.3(1b) isof orm was strongly up-regulated, whereas a more moderate increase was found for the Ca\textsubscript{a}1.3(1a) variant. Heterologous expression of Ca\textsubscript{a}1.3(1b) in HEK293 cells induced Ba\textsuperscript{2+} currents with properties similar to those found in Ca\textsubscript{a}1.2 (−/−) cardiomyocytes, suggesting that this isof orm constitutes a major component of the residual L-type calcium current in Ca\textsubscript{a}1.2 (−/−) cardiomyocytes. In summary, our results imply that calcium channel expression is dynamically regulated during heart development and that the Ca\textsubscript{a}1.3 channel may substitute for Ca\textsubscript{a}1.2 during early embryogenesis.

Two distinctive families of voltage-gated Ca\textsuperscript{2+} channels have been identified in the cardiac tissue of various vertebrates, namely the L-type and the T-type Ca\textsuperscript{2+} channels (1, 2). The principal α1 subunits of L-type Ca\textsuperscript{2+} channels expressed in embryonic hearts are encoded by three different genes, i.e., Ca\textsubscript{a}1.1 (α1C), Ca\textsubscript{a}1.2 (α1D), and Ca\textsubscript{a}1.3 (α1S) (2–5). Among these channels, Ca\textsubscript{a}1.2 is the predominantly expressed isoform in cardiomyocytes and is important for excitation-contraction coupling (3–7). Deletion of the Ca\textsubscript{a}1.2 gene results in embryonic death before day E14.5 (5), whereas deletion of the Ca\textsubscript{a}1.3 (8) and Ca\textsubscript{a}3.1 (9) genes does not significantly affect embryonic development. The Ca\textsubscript{a}1.3 Ca\textsuperscript{2+} channel contributes to the generation of the spontaneous action potentials in SA node cells by participating to diastolic depolarization (8, 10, 11). So far, no specific cardiac function has been reported for the faintly expressed α1S subunit during embryogenesis.

Two T-type Ca\textsuperscript{2+} channels have been detected in vertebrate heart, namely Ca\textsubscript{a}3.1 (α1G) and Ca\textsubscript{a}3.2 (α1H) (12, 13). T-type Ca\textsuperscript{2+} channels have been mainly studied in cardiac pacemaker cells and are thought to act together with the hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels (14) and the L-type Ca\textsuperscript{2+} channels in the generation of pacemaker potentials. There is evidence that in heart the T-type current also contributes to pathological processes such as ventricular hypertrophy, post-myocardial infarction, and arrhythmogenesis that occur during atrial fibrillation (15, 16). In contrast to these observations, Ca\textsubscript{a}3.1 knockout mice do not display a cardiac phenotype but reveal a lack of the burst firing of thalamocortical relay neurons and resistance to absence seizures (9).

Ca\textsuperscript{2+} channels have been extensively investigated by molecular, biochemical, and electrophysiological methods in heart cells (17–19). In contrast, there is only limited knowledge on the transcriptional levels of L- and T-type channels during heart development. We have shown previously that an intact Ca\textsubscript{a}1.2 gene is required for normal embryonic development after embryonal day E14 (5). Deletion of the cardiac calcium channel β2 gene results in embryonal death already after day E9.5 (20). In view of the relative late death of the Ca\textsubscript{a}1.2 (−/−) embryos, the early death of the β2 knockout mice was surprising. Furthermore, the analysis of Ca\textsubscript{a}1.2 (−/−) cardiomyocytes at day E12.5 revealed that rhythmic contractility depended on an L-type-like Ca\textsuperscript{2+} current with a low affinity for dihydropyridines (5). These results suggested that an unidentified L-type like calcium channel replaced the lacking Ca\textsubscript{a}1.2 channel during early embryogenesis.

In the present study, we compared the expression of six potential surrogate calcium channels by RT-PCR1 in murine cardiomyocytes derived from Ca\textsubscript{a}1.2-deficient embryos with that from wild type cells. We found major differences in the expression level of other calcium channel genes with a predominant up-regulation of a Ca\textsubscript{a}1.3 splice form that has the electrophysiological and pharmacological properties of the L-type current found in Ca\textsubscript{a}1.2 (−/−) cardiomyocytes.

**MATERIALS AND METHODS**

*Animals and Cardiomyocytes Isolation—*Wild type mice and Ca\textsubscript{a}1.2 (−/−) mice (5) were housed at 20 °C with a 12-h light/dark cycle. Individual embryos were obtained after breeding of wild type or heterozygous Ca\textsubscript{a}1.2 (±) mice. The hearts were dissected at embryonic day E9.5, E12.5, or E15.5 and were either used for cell isolation or quickly frozen and stored at −80 °C for RNA isolation. Embryonic cardiomyocytes were isolated as described (5). The dispersed cells were plated on 60-mm dishes for 48 h and then harvested for RNA isolation.

*RNA Isolation, First Strand cDNA Synthesis, and RT-PCR—*Frozen heart tissue or cultured cardiomyocytes were homogenized in Trizol LS

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1 The abbreviations used are: RT-PCR, reverse transcriptase PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK 293, Human embryonic kidney 293 (cells); HP, holding potential; NMDG, N-methyl-D-glucamine.
Reagents (Invitrogen), and total RNA was isolated according to the manufacturer's recommendations. Total RNA was then quantified by spectrophotometry. The RNA samples were not contaminated with DNA, which was checked by PCR in the absence of reverse transcriptase. First strand cDNA synthesis was carried out for 1 h at 42 °C in a 20-μl reaction mixture containing 4 μg of total RNA, 2 units of RNase H, 1.25 mM dNTP, 100 pmol of random hexamer primers, and 50 units of Superscript II reverse transcriptase (Invitrogen). The enzyme was inactivated by incubation at 90 °C for 5 min. The cDNAs were then stored at −80 °C. 

PCR was used to amplify the calcium channel cDNAs to determine the relative expression levels of the Cav1.3 (1a) or (1b) mRNA. The PCR products were then size-fractionated on agarose gels and stained with ethidium bromide. The gels were re-exposed to X-ray film to allow detection of the limiting fragment sizes. The size of the band for each product was determined and normalized to that of the GAPDH fragment. The identity of the PCR products was verified by DNA sequencing.

Electrophysiological experiments were performed as described earlier (5, 21). HEK 293 cells were transiently transfected with the expression vector for the Ca1.3 (1a) or (1b) subunit (0.2 μg per well) and the expression vectors for the β2 and the α1C-1 subunits (0.15 μg each per well) using LipofectAMINE according to the manufacturer's instructions (Invitrogen). The holding potential (HP) was −80 mV. Current-voltage relations (I-V) were recorded from −60 to 50 mV with 10-mV increments and a frequency of 0.2 Hz. Cumulative dose-response curves were measured using 2–3 different nisoldipine concentrations per cell. Trains of test pulses were to 0 or 10 mV for 40 ms with 0.1 Hz for I_{max} of native cells or to −10 or 0 mV for 100 ms with 0.2 Hz for I_{max} of expressed channels. I_{max} values were calculated by fitting the averaged dose-response curves to the Hill equation shown in Equation 1:

$$I_{max} = 1/(1 + ([nisoldipine]/IC_{50})^H)$$

where [nisoldipine] is the concentration of nisoldipine, IC_{50} is the half-blocking concentration, H is the Hill coefficient, f is the current measured at any concentration of nisoldipine, and I_{max} is the current measured in the absence of drug. The data for the I_{max} of wild type cardiomyocytes were fitted with a two-component Hill equation.

The intracellular solution contained 80 mM CsCl, 50 mM aspartic acid, 68 mM CsOH, 1 mM MgCl_2, 5 mM potassium-ATP, 1 mM CaCl_2, 10 mM HEPES, and 11 mM EGTA (pH 7.4). Seals were formed in a barium Tyrode solution of the composition 130 mM NaCl, 4.8 mM KCl, 5 mM BaCl_2, 1 mM MgCl_2, 5 mM glucose, and 5 mM HEPES (pH 7.4). The bath solution contained 130 mM N-methyl-D-glucamine (NMDG), 4.8 mM CaCl_2, 5 mM BaCl_2, 5 mM glucose, and 5 mM HEPES at pH 7.4 (NMDG Tyrode). Nisoldipine stock solution was 10 mM in ethanol. On each experimental day, nisoldipine was diluted from the stock solution into the NMDG Tyrode to the indicated concentrations.

**RESULTS**

Expression of Calcium Channel α1 Subunit mRNAs in Murine Fetal Heart and Cardiomyocytes—To analyze Ca^{2+} channel diversity in murine embryonic hearts, we determined the relative mRNA expression at day E9.5, a time point when the heart starts regular beating, at day E12.5, a time point when Ca_{1.2}-deficient embryos still develop, and at day E15.5, a time point when the heart is fully developed (22). To compare expression levels obtained from independent experiments, we normalized the data by using GAPDH as an internal standard. In agreement with others (1, 23, 24), we obtained amplicons for Ca_{1.1}, Ca_{1.2}, Ca_{1.3}, Ca_{3.1}, and Ca_{3.2} channels (Fig. 1A). No specific DNA fragments were detected for the R-type Ca_{2.3} and L-type Ca_{1.4} calcium channels. Transcripts of the L-type channels Ca_{1.1}, Ca_{1.2}, and Ca_{1.3} were present throughout the fetal heart development. At day E9.5 the expression levels of the three channels were nearly identical. In contrast to the Ca_{1.3} and Ca_{1.1} mRNAs, the Ca_{1.2} subunit was up-regulated −3-fold at day E15.5 (Fig. 1B). Ca_{1.2} becomes the predominant L-type channel isof orm in late developmental stages. In isolated embryonic cardiomyocytes, up-regulation of Ca_{1.2} was even slightly more pronounced than in total heart at E15.5 (not shown). In general, the changes in mRNA levels were more pronounced in cardiomyocytes than in total heart tissue, which may be caused by various expression levels in multiple cardiac cell types. Whereas in total heart the Ca_{3.1} T-type Ca^{2+} channel subunit could be consistently found at all three time points investigated, Ca_{3.2} was barely detectable at all devel-

**TABLE I**

| Name          | Primer      | 5'-Location (bp) | Sequence (5'-3')                                      | Size (bp) |
|---------------|-------------|-----------------|------------------------------------------------------|-----------|
| Ca_{1.1}      | F           | 1235            | GCGAGATCTAGTGGACACTTGAGAC                             | 200       |
| L06234        | R           | 1434            | GATCCACGCGCATAGATAGAGAC                               | 477       |
| Ca_{1.2}      | F           | 3527            | CAGAGGGTTGGAGGAAAGCCCA                                 | 316       |
| L01776        | R           | 3842            | CTGGCCGCGGAGCTCCTGGGG                                  | 555       |
| Cav_{1.3}     | R           | 3217            | GGTCGCCACTCTCTTGGG                                    | 279       |
| AJ437292      | R           | 3771            | GCTGCTGAACTCCTTGGG                                    | 328       |
| Cav_{3.1}     | F           | 2090            | AGAGAGAATTCTCAATAAGCT                                  | 590       |
| AJ1012569     | R           | 3276            | GCTGCTGAACTCCTTGGG                                    | 649       |
| Cav_{3.2}     | F           | 3105            | AGAGAGAATTCTCAATAAGCT                                  | 590       |
| AF061946      | R           | 3432            | GCTGCTGAACTCCTTGGG                                    | 649       |
| Cav_{1.3}(1a) | Dupa01      | −45             | AGCAAYSTGCYCTCATGCACAGYTAGA                           | 316       |
| AJ437291      | RACE12      | 546             | GCAGTACATACCTCATATTTCTCGAATAAAGAT                    | 450       |
| Cav_{1.3}(1b) | RACE20      | −31             | GCAGTACATACCTCATATTTCTCGAATAAAGAT                    | 450       |
| AJ437292      | RACE29      | 618             | GCAGTACATACCTCATATTTCTCGAATAAAGAT                    | 450       |
Therefore, we determined the expression of calcium channel proteins in the heart during early embryogenesis. Calcium channel protein, because their open reading frame is disrupted by the introduction of a premature stop codon in exon 3 (5). These transcripts are not translated into a functional calcium channel protein, because their open reading frame is disrupted by the introduction of a premature stop codon in exon 3 (5). These transcripts are not translated into a functional calcium channel protein. Deletion of the Cav1.2 gene increased the expression levels of three other channels, Ca_{1,1}, Ca_{1,3}, and Ca_{3,1} at day E9.5 (Fig. 2B). The mRNA of the Ca_{3,1} channel was not detected in wild type cardiomyocytes but was present in Ca_{1,2}(-/-) cells at day E9.5 (Fig. 2B). At day E12.5, similar levels of mRNA were detected for the Ca_{1,1} and Ca_{3,1} channels. In contrast, the expression of Cav1.3 changed dramatically. At days E9.5 and E12.5, the mRNA of this channel was up-regulated 4-fold in Ca_{1,2}(-/-) cardiomyocytes. To test this hypothesis, we expressed Cav1.3(1a) and Cav1.3(1b) in HEK 293 cells and compared the expressed currents with currents from wild type cardiomyocytes but was present in Ca_{1,2}(-/-) cells at day E9.5 (Fig. 2B). At day E12.5, similar levels of mRNA were detected for the Ca_{1,1} and Ca_{3,1} channels. In contrast, the expression of Cav1.3 changed dramatically. At days E9.5 and E12.5, the mRNA of this channel was up-regulated 4-fold in Ca_{1,2}(-/-) cardiomyocytes. To test this hypothesis, we expressed Cav1.3(1a) and Cav1.3(1b) in HEK 293 cells and compared the expressed currents with currents from wild type cardiomyocytes but was present in Ca_{1,2}(-/-) cells at day E9.5 (Fig. 2B). At day E12.5, similar levels of mRNA were detected for the Ca_{1,1} and Ca_{3,1} channels. In contrast, the expression of Cav1.3 changed dramatically. At days E9.5 and E12.5, the mRNA of this channel was up-regulated 4-fold in Ca_{1,2}(-/-) cardiomyocytes.
expressing Cav1.3(1b) from cells only containing endogenous current was not an L-type current. To safely separate cells appeared at an HP of −80 mV, not sensitive to the dihydropyridine nisoldipine and disappear in pcDNA3 vector-transfected cells. The current was detected in murine embryonic hearts at day E12.5. p.c., post coitum.

C. Western blotting of CaV1.2, CaV1.3, and β-actin in murine embryonic hearts day E12.5.

and β subunits has been reported in cardiac muscle (25). In initial experiments CaV1.3b was coexpressed with the β2a or β3 subunit, but in the presence of the β2a subunit only current densities below 1.0 pA/pF were obtained. Therefore we investigated the CaV1.3 subunit in further experiments in the presence of the β3 subunit. The coexpression of β3 with CaV1.3 will also facilitate comparability of our results with those of others (26).

Both isoforms induced L-type barium currents with slow inactivation kinetics in 55% (CaV1.3(1a)) and 16% (CaV1.3(1b)) of transfected HEK 293 cells (Fig. 4, A and B). Some of the untransfected HEK 293 cells (control cells) showed a small rapid inactivating inward current with a current density at 0 mV of 0.64 ± 0.08 pA/pF (n = 3). A similar current could be detected in pcDNA3 vector-transfected cells. The current was not sensitive to the dihydropyridine nisoldipine and disappeared at an HP of −40 mV, suggesting that this endogenous current was not an L-type current. To safely separate cells expressing CaV1.3(1b) from cells only containing endogenous current, only cells showing a slowly inactivating current and a current density above 1.0 pA/pF were considered for analysis.

With 5 mM Ba2+ as charge carrier, the current density of CaV1.3(1a)- and CaV1.3(1b)-transfected cells was 11.8 ± 2.0 pA/pF (n = 30) and 1.9 ± 0.24 pA/pF (n = 25), respectively. The I-V curves indicated that the current through the CaV1.3 splice forms activated negative from −40 mV and were maximal between −10 mV and 0 mV (Fig. 4C). This result is in good agreement with the L-type current described in CaV1.2 (−/−) cardiomyocytes (5).

I

Ba of wild type cardiomyocytes was blocked by nisoldipine with IC50 values of 0.1 and 3.9 μM. In contrast, I

Ba currents of CaV1.2 knockout cells were blocked with an IC50 of 1.1 μM (Fig. 4E). The IC50 values for the nisoldipine block of I

Ba induced by the heterologously expressed CaV1.3 splice variants differed from each other, being 0.1 μM (CaV1.3(1a)) and 0.41 μM (CaV1.3(1b)) (Fig. 4D). The latter value is close to that found in CaV1.2 (−/−) cardiomyocytes, suggesting that CaV1.3(1b) channels could be responsible for the L-type like Ca2+ current in CaV1.2 knockout cells.

**DISCUSSION**

In this study, we investigated for the first time the developmental expression levels of all cardiac calcium channel α1 subunits in murine fetal hearts. In agreement with previous electrophysiological data (27), we observed an up-regulation of three subunits underlying L-type calcium currents (CaV1.1, CaV1.2, and CaV1.3). Up-regulation of the CaV1.2 subunit was much more pronounced than that of the other two subunits. This finding emphasizes the particular importance of CaV1.2 for heart function, especially during late fetal stages and in the adult heart. In contrast, in early cardiac developmental stages, when the expression levels of CaV1.2 are low, mechanisms that do not necessarily include the CaV1.2 channel may control heart contraction (28, 29). Like CaV1.2, the CaV1.1 and CaV1.3 subunits are also significantly up-regulated between days E9.5 and E12.5. In contrast to CaV1.2, the expression levels of the two latter subunits increase only slightly after day E12.5.
Taken together, these findings imply that Cav_1.1 and Cav_1.3 possibly fulfill an unknown role in early heart development that may change at later stages of cardiac development. A function for Cav_1.1 has not been reported, but for Cav_1.3 it has been shown that this channel is important for the generation of spontaneous action potentials in sino-atrial node cells of the adult heart (10, 11).

The expression levels of the two cardiac T-type calcium channels differ profoundly from each other during heart development. Cav_3.2 expression levels were very low. In isolated cardiomyocytes and in heart tissue this subunit could not be detected at day E15.5, suggesting that Cav_3.2 does not play a substantial role in adult heart muscle. However, we cannot exclude that Cav_3.2 may be expressed only in a subset of heart cells (e.g. cells of the conduction tissue) and, therefore, was not detected in whole heart preparations. The low but clearly measurable expression of Cav_3.2 at days E9.5 and E12.5 points to a possible role of this subunit in early heart development. In agreement with previous studies (6, 13), the Cav_3.1 mRNA was consistently detected in the developing heart, indicating that Cav_3.1 is the major cardiac T-type calcium channel isoform.

As we pointed out, mice deficient for Cav_1.2 die in utero before day E14.5. The results of this study indicate that the loss of this key subunit increases the mRNA levels of Cav_1.3, Cav_1.1, and Cav_3.1. Although the increase of the latter two subunits is only moderate, the mRNA and the protein levels of the Cav_1.3 subunit are profoundly up-regulated. RT-PCR analysis indicated that the increase in Cav_1.3 expression is mainly due to the up-regulation of the Cav_1.3(1b) isoform of this channel. The electrophysiological and pharmacological properties of the Cav_1.3(1b) subunit heterologously expressed in HEK293 cells are in good agreement with that of the residual L-type calcium current found in Cav_1.2 (−/−) cardiomyocytes. These results strongly suggest that Ca^{2+} influx through Cav_1.3(1b) is a major entry pathway in Cav_1.2 (−/−) cardiomyocytes.

The functional relevance of Cav_1.3 up-regulation is evident. Most likely, the up-regulation of Cav_1.3 constitutes a compensatory mechanism of the cell to rescue the loss of Cav_1.2 function, strongly suggesting the importance of Cav_1.2. However, the compensatory increase in the Cav_1.3 subunit is not sufficient to allow development of the embryo beyond day E14. As shown in this report, the Cav_1.2 mRNA level increases at this time, presumably because only this channel provides the necessary calcium ions for cardiac contraction. The importance of the cardiac Cav_1.2 calcium channel during embryogenesis has also been demonstrated recently by another study showing that the inactivation of the cardiac β2 subunit resulted in an early death at E11.5 (20). It is likely that the early death of the β2 subunit (−/−) mice is caused by an ER-retention of the Cav_1.2 and the Cav_1.3 subunit, because it was shown that the β2 subunit suppresses an ER-retention signal present in the α1 subunits (30).

The results of this study add to the growing evidence that the different α1 subunits of the high voltage-activated calcium channels can substitute for each other in certain, but not all, functions. Our results indicate that there is a transcriptional cross-talk between the Cav_1.2 and Cav_1.3 calcium channel genes. A cross-talk between these two calcium channels has also been described in pancreatic β cells of Cav_1.3-deficent mice (31). Furthermore, a compensatory increase in the expres-

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