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Expression of Ca^{2+} Channel Subunits During Cardiac Ontogeny in Mice and Rats: Identification of Fetal α_{1C} and β Subunit Isoforms

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Abstract Functional cardiac L-type calcium channels are composed of the pore-forming α_{1C} subunit and the regulatory β_2 and α_2/δ subunits. To investigate possible developmental changes in calcium channel composition, we examined the temporal expression pattern of α_{1C} and β_2 subunits during cardiac ontogeny in mice and rats, using sequence-specific antibodies. Fetal and neonatal hearts showed two size forms of α_{1C} with 250 and 220 kDa. Quantitative immunoblotting revealed that the rat cardiac 250-kDa α_{1C} subunit increased about 10-fold from fetal days 12–20 and declined during postnatal maturation, while the 220-kDa α_{1C} decreased to undetectable levels. The expression profile of the 85-kDa β_2 subunit was completely different: β_2 was not detected at fetal day 12, rose in the neonatal stage, and persisted during maturation. Additional β_2-stained bands of 100 and 90 kDa were detected in fetal and newborn hearts, suggesting the transient expression of β_2 subunit variants. Furthermore, two fetal proteins with β_4 immunoreactivity were identified in rat hearts that declined during prenatal development. In the fetal rat heart, β_4 gene expression was confirmed by RT-PCR. Cardiac and brain β_4 mRNA shared the 3 prime region, predicting identical primary sequences between amino acid residues 62–519, diverging however, at the 5 prime portion. The data indicate differential developmental changes in the expression of Ca^{2+} channel subunits and suggest a role of fetal α_{1C} and β isoforms in the assembly of Ca^{2+} channels in immature cardiomyocytes. J. Cell. Biochem. 76:695–703, 2000.

Key words: calcium channel; α_{1C} subunit; β subunit; heart development; rat; mouse

Transsarcolemmal influx of Ca^{2+} into cardiac cells via voltage-gated L-type Ca^{2+} channels plays a critical role in cardiac contractile function. The local increase in Ca^{2+} concentration triggers the opening of sarcoplasmic reticular ryanodine receptor/Ca^{2+} release channels. In mature myocardium, large quantities of Ca^{2+} are released from the SR, leading in turn to cell contraction [Barry and Bridge, 1993]. By contrast, immature mammalian myocardium lacks the well-developed SR [Anderson, 1989] and the Ca^{2+}-induced Ca^{2+} release [Fabiato, 1982]. Consequently, results from several studies have suggested that the contraction of immature cardiomyocytes is more dependent on the movement of Ca^{2+} across the sarcolemma compared as with mature cardiomyocytes [for review, see Nakanishi, 1992].

Functional Ca^{2+} channels are oligomeric proteins consisting minimally of an α_1 subunit along with accessory α_2/δ and β subunits [Perez-Reyes and Schneider, 1994]. The α_1 subunit resides in the membrane and forms the ion-conducting pore. Cardiac myocytes express the

Abbreviations used: SR, sarcoplasmic reticulum; PKA, protein kinase A; DHP, 1,4-dihydropyridine(s); IgG, immunoglobulin G; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcription polymerase chain reaction; I_{Ca}, Ca^{2+} inward current.

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α_{1C} gene coding for the 242-kDa DHP receptor/Ca^{2+} channel [Mikami et al., 1989]. Biochemical studies indicated the presence of two size forms of the cardiac DHP receptor protein: the full-length translation product and the C-terminal truncated isoform [Haase et al., 1991; Gao et al., 1997]. Further heterogeneity is introduced by the expression of developmentally regulated α_{1C} isoforms, which differ in the third membrane-spanning region of motif IV [Diebold et al., 1992]. The β subunit binds to the α_1 via conserved binding domains [Pragnell et al., 1994; De Waard et al., 1994] and acts to modulate fundamental channel properties such as DHP binding and channel kinetics in a β isoform-specific manner [Perez-Reyes and Schneider, 1994]. Four genes coding for β subunits have been identified (β_1–β_4), several of which express splice variants [Castellano and Perez-Reyes, 1994]. In cardiac muscle, the β_2 subunit isoforms is preferentially expressed. Colocalization [Gao et al., 1997] and coprecipitation [Haase et al., 1993, 1996; Pichler et al., 1997] of the α_{1C} and β_2 indicated that both subunits constitute native Ca^{2+} channels in the adult mammalian heart.

Developmental changes in Ca^{2+} channel expression and function have been demonstrated in numerous studies [for review, see Renaud et al., 1989]. DHP binding experiments suggested low Ca^{2+} channel expression in immature hearts, which increased during cardiac maturation [Navaratnam and Khatter, 1991; Wibo et al., 1991]. In addition, electrophysiological data demonstrated a progressive increase in Ca^{2+} inward current throughout fetal and postnatal life [Wetzel et al., 1993; Huynh et al., 1992]. It remains unclear how the immature cardiomyocyte with low Ca^{2+} channel density is able to maintain functional contractility, which, at this stage, directly depends on the influx of external Ca^{2+}. A possible answer is that intrinsic channel properties change during development due to the expression of structurally different Ca^{2+} channels. To address this issue, we studied the temporal expression profile of the Ca^{2+} channel α_{1C} and β_2 subunits on the protein level, using sequence-directed antibodies. Our results indicate a noncoordinated expression pattern, in which the α_{1C} expression precedes the β_2. Moreover, the data demonstrate for the first time the expression of fetal α_{1C} and β subunit isoforms.

MATERIALS AND METHODS

Materials

Unless otherwise noted, all reagents were obtained from Sigma, Deisenhafen, Germany. Enhanced chemiluminescence kit (ECL) was from Amersham. Rats and mice were purchased from a local source. The strains were Wistar rats (Schö: Wist) and Black 6 mice (PG 129, B6), respectively.

Tissue Preparation

Rats and mice were euthanized and the hearts removed and immediately frozen in liquid nitrogen. Tissues were stored at −70°C. Proteins were extracted from frozen tissue specimens by homogenization with a motor-driven glass-Teflon homogenizer in sodium dodecyl sulfate (SDS)-sample buffer (5% SDS, 50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 75 mM urea, 60 mM β-mercaptoethanol) and denatured for 2 min at 95°C. Fetal mouse hearts were removed under microscopic control and immediately solubilized with 50 µl of SDS-sample buffer. The SDS-extracted proteins were further processed and determined as previously described [Haase and Morano, 1996].

Production and Purification of Antibodies

New Zealand white rabbits were immunized with synthetic peptides coupled to carriers as previously described [Haase et al., 1993; McEnery et al., 1997]. Antigenic epitopes comprised the amino acid residues 799–813 (EEEEKERKKLARTASPEKK) of the rabbit cardiac α_{1C} cDNA [Mikami et al., 1989], amino acids 597–606 (EWNRDVYIRQ) of the rabbit cardiac β_2 cDNA [Hullin et al., 1992] and amino acids 458–474 (ENYHNERARKSRNRLS) of the rat brain β_4 cDNA [Castellano et al., 1993]. The antibodies were affinity-purified on antigen columns [Calovini et al., 1995] and have been characterized previously: α_{1C}, [Hoch et al., 1998; Gollasch et al., 1998], β_2 [Haase et al., 1996; Pichler et al., 1997; McEnery et al., 1997; Safayhi et al., 1997; Vance et al., 1998] and β_4 [Vance et al., 1998]. For comparison, the β_{1b}- and β_3-specific were employed, their staining pattern was described previously [Pichler et al., 1997; McEnery et al., 1997; Vance et al., 1998]. Each dilution of the β-isoform-specific antibodies used in the experiments was tested on a control preparation of rat brain and gave the expected characteristic staining pattern. Con-

trol staining for the α_{1C} subunit was performed using purified pig cardiac Ca^{2+} channels [Haase et al., 1991].

SDS-PAGE and Immunoblot Analysis

Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 6.5% resolving gel and transferred to nitrocellulose filters according to standard procedures. The transfers were incubated with the affinity-purified primary antibodies at a concentration of 0.5–1 µg IgG/ml for 90 min and the secondary peroxidase-conjugated anti-rabbit antibody. Immunoreactive protein bands were visualized by ECL reaction kit, using an x-ray film.

RNA Preparation and cDNA Synthesis

Total RNA was prepared from frozen tissue of fetal rat hearts (day 14, day 21 is full-term) and from adult rat brain by the guanidium thiocyanate procedure [Chomczinski and Sacchi, 1987]. Total RNA in 1-µg aliquots was converted to first-strand cDNA with 10 pmol of random hexamers and superscript reverse transcriptase (RT) (1 U, Gibco-BRL) in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM dNTP, and 10 mM dithiothreitol.

PCR and Sequencing

Oligodenucleotide primers were synthesized and purified by the Oligonucleotide Synthesis Core of the Max-Delbrück-Centrum (BioTez, Berlin). The primers were designed to match sequences of the rat brain Ca^{2+} channel β_4 subunit [Castellano et al., 1993]. Polymerase chain reaction (PCR) reactions contained 5 µl of RT reaction as template, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 20 µM each dATP, dCTP, dGTP, dTTP, 1 µm each primer, and 1.5 U *Taq*-DNA polymerase (Gibco-BRL). Reactions were performed with a thermocycler for 32 cycles under the following conditions: 94°C (1 min); 60°C (1 min); 72°C (90 s); final extension 72°C (10 min). The amplified cDNA fragments were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. Sequence analysis of the PCR products was performed commercially (InVitek, Berlin) by an ABI 373 DNA sequencer (Perkin-Elmer), using the ABIPRISM dye terminator cycle sequencing method. A total of 10–20 ng of gel resolved PCR fragments and 3.2 pmol/L of each primer (forward, reverse) in a total volume of 5 µl. The reactions were performed on a Gene Amp PCR system 2400 (Perkin-Elmer).

Cloning of Polymerase Chain Reaction Products

Amplified fragments of a 30-cycle PCR were cloned into pCR 2.1 vector, using the TA Cloning Kit (Invitrogen) according to the manufacturer's protocol. Single white colonies and their plasmid DNA were isolated, and inserted PCR products were analyzed by restriction with *Eco*R1 and sequenced as described above.

RESULTS

Expression of Ca^{2+} Channel α_{1C} and β_2 Subunits During Mouse Heart Development

To study the temporal expression of L-type Ca^{2+} channel constituents during cardiac maturation, we raised anti-peptide antibodies against unique sequences of α_{1C} and β_2 subunits. Immunoblots of fetal (day 15 of gestation, day 20 is full term), neonatal (postnatal days 1–3), and adult mouse hearts are demonstrated in Figure 1. The anti-α_{1C} antibody directed against an internal sequence of class C α_1 subunits identified a 250-kDa protein in the experimental groups, indicating the presence of the full-length α_{1C} protein. In immature hearts of fetal and neonatal mice, additional 220-kDa α_{1C}-immunostaining was observed (Fig. 1, middle, lanes 1, 2). Surprisingly, α_{1C} staining intensity of both forms was highest in fetal hearts, de-

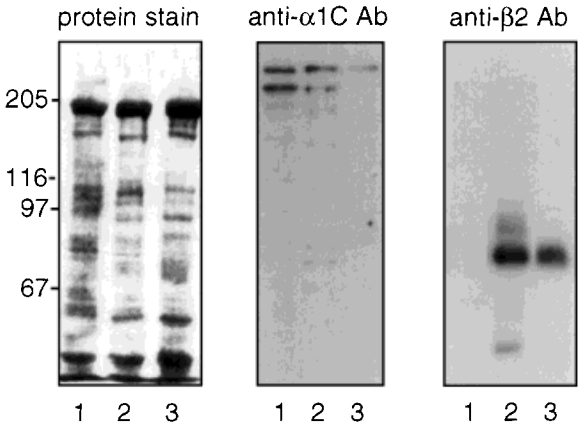

Fig. 1. Detection of α_{1C} and β_2 subunits of the L-type Ca^{2+} channel in developing mouse hearts. Heart tissue was solubilized with SDS-sample buffer. Samples were subjected to SDS-PAGE and Western blotting. The nitrocellulose transfers were stained for protein with Ponceau S (left) and probed with sequence-directed antibodies (Ab) against the α_{1C} and β_2 subunits as indicated. 1, fetal day 15; 2, postnatal days 1–3; 3, adult heart.

creasing gradually with cardiac development. This result was not due to remarkable differences in the concentrations of analyzed muscle proteins, as depicted by parallel protein staining (Fig. 1, left). Densitometric analysis of α_{1C} staining demonstrated a significant (about 8-fold) reduction of 250-kDa α_{1C} in adult hearts compared with fetal hearts, when normalized to the 200-kDa myosin heavy chain band known as prominent cardiac muscle protein. By contrast, the expression of the Ca^{2+} channel β_2 subunit was found to be upregulated. The affinity-purified anti-β_2 antibody reacted strongly with a 85-kDa protein in neonatal and adult, but not in fetal mouse heart (Fig. 1, right). This apparent molecular mass is consistent with the migration of β_2 subunits of the mammalian heart and brain [Haase et al., 1996; Pichler et al., 1997; McEnery et al., 1997; Gollasch et al., 1998; Safayhi et al., 1997; Vance et al., 1998]. An additional diffuse β_2 immunostaining around 90 kDa was only visible in the neonatal state.

Expression Profile of Ca^{2+} Channel α_{1C} and β_2 Subunits During Rat Heart Development

Developing rat hearts were then used to analyze the temporal expression of Ca^{2+} channel α_{1C} and β_2 subunits in more detail. The rat model permitted normalization of channel components to the concentration of total cardiac proteins. This mode of evaluation is important, since myosin expression by itself increased with cardiac development. Identical protein concentrations from either fetal hearts at days 12, 14, and 20 of gestation (day 21 is full term) or hearts at different stages of postnatal maturation (neonatal days 1–3, until 6 weeks) were probed on Western blots with the α_{1C}- and β_2-specific antibodies. Similar to the mouse model, we observed two size α_{1C} forms in fetal and neonatal rat hearts, while adult animals showed solely the 250-kDa full-length α_{1C} protein (Fig. 2, left). Relative densitometry measurements indicated that the 250-kDa α_{1C} increased about 10-fold from fetal days 12–20, persisted in newborn hearts, and decreased about 8-fold with adult maturation (Fig. 2, left). The 220-kDa α_{1C} isoform appeared as a prominent isoform early in cardiac development. Its expression was strongly downregulated after birth (Fig. 2, left, lower).

In the adult heart, the 85-kDa β_2 subunit reacted strongly with the β_2 antibody (Fig. 2, right), corresponding to the β_2-immunorecogni-

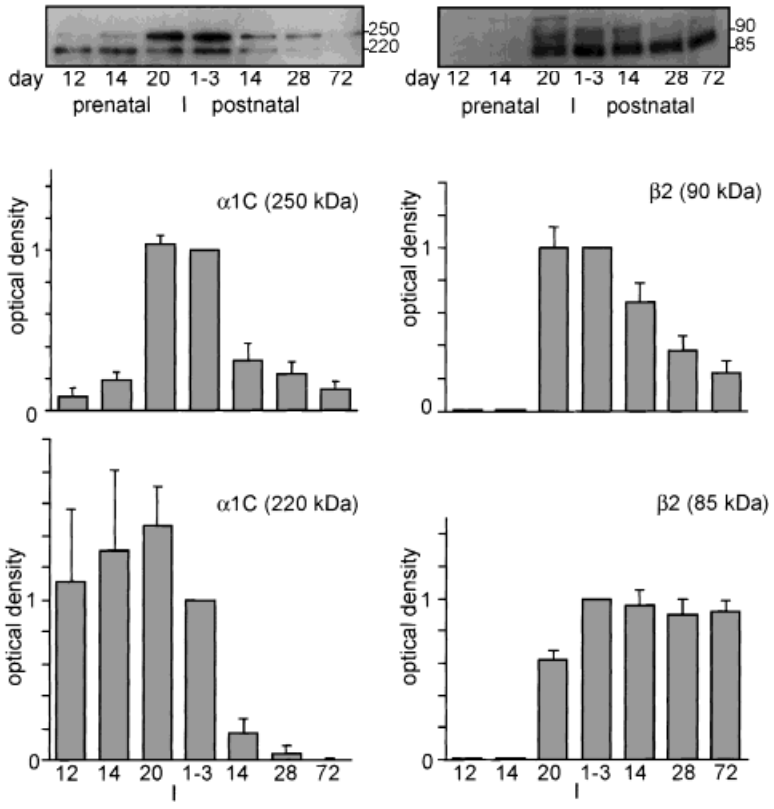

Fig. 2. Expression profiles of α_{1C} and β_2 subunits of the L-type Ca^{2+} channel during rat heart development. SDS-extracted proteins from rat hearts at fetal day 12, 14, and 20, from newborn (days 1–3), and from different days of postnatal maturation were probed on Western blots with the anti-α_{1C} (**top left**) and the β_2-directed antibodies (**top right**). Equal amounts of proteins (20 μg) were analyzed in 3–6 independent experiments. Bar graphs depict arbitrary units obtained by normalization of the optical densities of immunostained bands related to the staining intensity of respective bands in newborn hearts.

tion of adult mouse heart (Fig. 1). Interestingly, the β_2 protein was below the detection limit at embryonic days 12 and 14. It became visible at fetal day 20, rose to the neonatal stage from 0.6 to 1.0 relative units, and persisted at this expression level (Fig. 2, right, lower). The β_2-directed antibody recognized two additional proteins in the early stages of cardiac development: a broad 90-kDa and a sharp 100-kDa band. The 90-kDa band was visible between fetal day 20 and postnatal day 14 (Fig. 2, right), while the 100-kDa β_2 band was detected in fetal and newborn hearts (Figs. 2, 3).

The transient expression of β_2 variants in immature hearts was confirmed by analyzing higher amounts of fetal and neonatal rat heart proteins. The detection limit of both the 85- and the 90-kDa β_2 isoform was reached at fetal day 15, since a weak diffuse staining was observed in the respective molecular-mass range (Fig. 3A). Their abundance increased tremendously between fetal day 15 and early neonatal state, while the 100-kDa β_2 variant reacted already strongly at fetal day 15 (Fig. 3A).

β_4-Immunoreactivity in Fetal Rat Hearts

The extremely low β_2 expression level in early fetal hearts led us to investigate whether other β subunit isoforms may substitute for β_2. Immunoblots of rat hearts at different developmental stages were probed with β-isoform-specific antibodies against the β_{1b}, β_3, and β_4 subunits.

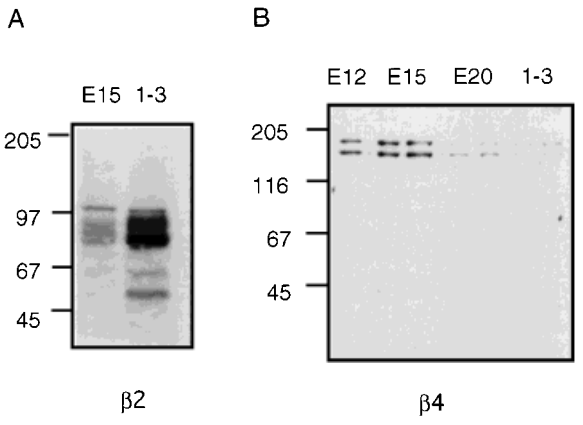

Fig. 3. Immunorecognition pattern of β_2- and β_4-subunit-specific antibodies in fetal and newborn rat hearts. **A:** A total of 60 μg of rat heart proteins from fetal day 15 and postnatal days 1–3 was probed on Western blots with the β_2-specific antibody. **B:** A total of 20 μg of cardiac proteins from fetal days 12, 15, and 20, and from newborn (1–3) rats was probed with the β_4-specific antibody. Note that two different preparations were used for E15, E20, 1–3.

While β_{1b}- and β_3-directed antibodies showed no specific immunostaining (data not shown), the β_4-specific antibody recognized two proteins of 170 kDa and 150 kDa on immunoblots of rat hearts at fetal days 12 and 14 (Fig. 3B). This apparent molecular mass differed markedly from that of the β_4 in brain migrating at 65 kDa [Vance et al., 1998]. Interestingly, there were reciprocal expression profiles of β_4 and β_2 in rat heart: while the β_4 immunostaining disappeared at fetal day 20 (Fig. 3B), β_2 expression could be detected (Fig. 2, left).

Identification of Ca^{2+} Channel β_4 Transcripts in Fetal Rat Hearts by RT-PCR

To identify transcripts of the β_4 gene in fetal hearts, homology-based RT-PCR was performed with oligodenucleotide primers matching the cDNA sequence of rat brain Cchb4 [Castellano et al., 1993] (Table I). Fetal heart cDNA was obtained by reverse transcription of mRNA extracted from rat hearts at day 14 of gestation. In initial experiments, we used PCR primers spanning the N-terminal (f1/r1; 744-bp) and the C-terminal (f2/r2; 718-bp) domains of Cchb4 cDNA (Fig. 4). The C-terminal 718-bp fragment was easily identified by ethidium bromide staining after a 32-cycle PCR, while the N-terminal region was not amplified (Fig. 4A). Control experiments with brain mRNA showed that the f1/r1 primer combination produced the expected 944-bp cDNA fragment (data not shown), suggesting that cardiac and brain β_4 mRNA diverged at the 5 prime portion. We then chose primers downstream to f1, designated f3, f4, and f5 (Table I). Their location relative to the Cchb4 cDNA is depicted in Figure 4. PCR reactions using these primers gave multiple products (Fig. 4B).

To determine the basis for the size differences, RT-PCR products were cloned and sequenced. Four distinct cDNA were identified and designated 4a, 4d1, 4d2, and 4i (Fig. 5). The nucleotide sequences obtained for the 4a form were virtually identical to rat brain Cchb4 (L02315), except for two differences observed at bases 820 (A instead of G in L02315) and 874 (G instead of A in L02315), neither of which altered the deduced amino acid sequence. Thus, cardiac and brain β_4 mRNAs shared the 3 prime region, predicting identical primary sequences between amino acid residues 62–519 (corresponding to Cchb4).

TABLE I. Homology-Based RT-PCR to Amplify β_4 cDNA Fragments From Fetal Rat Heart

Primer combinations	Forward[a]	Reverse[b]	Expected product (bp)
f1/r1	ATGTCGTCCTCCTACGCCAAG 203–223	GATGGTGTCTGCATCGAGAAC 1127–1147	944
f2/r2	GATCCAGCCTAGCGGAAGTAC 1056–1076	GCAAATTGTCAACAGATGATG 1794–1814	767
f3/r1	GATGTCTCTTTGGAAGAGGACC 386–407	1127–1147	761
f3/r3	386–407	TGTGCTC**T**GTCACTTTCTGC# 808–827[c]	441
f4/r1	CATACGGATTCAACAGGAACAG 676–697	1127–1147	471
f5/r1	GCAAAACAGAAGCAGAAAGTG 797–817	1127–1147	350

[a]Corresponding to nucleotide no. in Cchb4.
[b]Complementary to nucleotide no. in Cchb4 (Castellano et al. [1993], GenBank accession L02315).
[c]T in boldface is complementary to our fetal rat heart sequence.

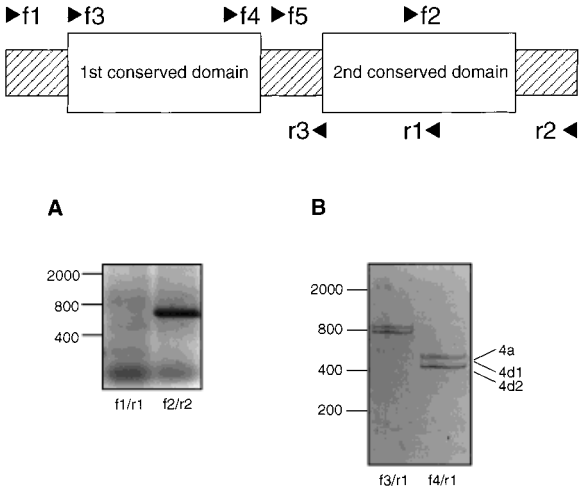

Fig. 4. Identification of Ca^{2+} channel β_4 transcripts in the fetal rat heart by RT-PCR. **Top:** Schematic representation of β subunits showing the two conserved domains and the variable regions (hatched boxes). The location of forward (f1–f5) and reverse (r1–r3) primers is indicated (sequences are given in Table I). **Bottom:** PCR reaction products from reverse transcribed fetal rat heart RNA using the indicated primers. Fragments were resolved on 1% agarose gel (**A**) or 8% polyacrylamide gel (**B**) and stained with ethidium bromide.

In two aberrant fetal rat heart cDNA species, we observed a 20-nucleotide deletion corresponding exactly to the renal β_{4d} species reported by Yu et al. [1995]. These transcripts were therefore designated 4d1 and 4d2 (Fig. 5). Deletion of the 20-nucleotide exon coding for the amino acid sequence AKQKQK leads to a shift in the open reading frame and premature truncation. Thus, a second downstream deletion of 59 nucleotides (corresponding to 899–957 in Cch4b), which discriminates between our 4d1 and 4d2 transcripts, is not expected to encode an additional truncated protein. The 4d2 transcript could be assigned to the smallest cDNA species obtained with the f4/r1 primers (Fig. 4B) by sequencing the PCR product. The upper bands comprised the 4a and 4d1 forms (Fig. 4B). These data suggest that in fetal rat heart, the β_{4a} transcript is less abundant than the deleted β_{4d1} and β_{4d2} variants. A fourth aberrant cDNA, designated 4i, was obtained using f5/r1 primers, which contained a large inserted sequence of about 300 nucleotides between the variable region coding for AKQKQK and the second conserved domain. This nucleotide sequence predicts, however, a truncated protein as well (Fig. 5).

DISCUSSION

This article reports on the expression pattern of L-type Ca^{2+} channel subunits during the fetal and postnatal periods of cardiovascular development in rodents. Consistent with previous findings on the subunit composition of native cardiac L-type Ca^{2+} channels [Gao et al., 1997; Haase et al., 1996; Cens et al., 1996], we demonstrate that mature hearts contain the channel-forming α_{1C} subunit together with the β_2 subunit isoform. There are two novel findings in this study: (1) different developmental expression profiles displayed by α_{1C} and β_2 subunits of rodents, and (2) expression of fetal isoforms of both α_{1C} and β subunits.

Acquisition of Cardiac Ca^{2+} Channels During Fetal Development

It is generally assumed that the sarcolemma L-type Ca^{2+} channel increases in conjunction with the perinatal and postnatal maturation of cardiac function [Renaud et al., 1989; Navaratnam and Khatter, 1991; Wibo et al., 1991; Wetzel et al., 1993; Huynh et al., 1992; Osaka et al., 1991]. On the basis of relative densitometric measurements of immunostained channel components, we found an increase in α_{1C} and β_2 protein expression from the fetal to neonatal

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4a,d1,d2,i :DVSLEEDREAIRQEREQQAAIQLERAKSKPVAFAVKTNVSYCGALDEDVPVPSTAISFDA    60
4a,d1,d2,i :KDFLHIKEKYNNDWWIGRLVKEGCEIGFIPSPLRLENIRIQQEQKRGRFHGGKSSGNSSS   120

4a         :SLGEMVSGTFRATPTTTAKQKQKVTEHIPPYDVVPSMRPVVLVGPSLKGYEVTDMMQKAL   180
4d1,4d2    :.................-------DRAHSSV*        144
4i         :.................AKQKQKVVSCVSFLNSC*     154

4a         :FDFLKHRFDGRISITRVTADISLAKRSVLNNPSKRAIIERSNTRSSLAEVQSEIERIFEL   240
4a         :ARSLQLVVLDADTINHPAQLIKTSLAPIIVHVKVSSPKVLQRLIKSRGKSQSKHLNVQLV   300
4a         :AADKLAQCPPEMFDVILDENQLEDACEHLGEYLEAYWRATHTSSSTPMTPLLGRNVGSTA   360
4a         :LSPYPTAISGLQSQRMRHSNHSTENSPIERRSLMTSDENYHNERARKSRNRLSSSSQHSR   420
4a         :DHYPLVEEDYPDSYQDTYKPHRNRGSPGGCSHDSRHRL*                        458
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Fig. 5. Amino acid sequence alignment of PCR products from fetal rat heart. Overlapping cDNA fragments were amplified using the primers given in Table I and Fig. 4. The PCR products were either directly sequenced or cloned into pCR 2.1 vector, and the plasmid DNA was sequenced. Asparagine at position 1 derived with f3/r3 primers corresponds to ^{62}D in Cchb4 [Castellano et al., 1993]. Dots indicate identity; dashes indicate gaps; asteriks indicate position of translational stop signal. Amino acids comprising the antigenic epitope for the β_4-directed antibody are in boldface.

stages of cardiac development in parallel to the myogenesis. The onset of α_{1C} and β_2 protein expression was, however, different. While the channel-forming α_{1C} subunit was identified at days 12 and 15 in fetal rat and mouse hearts, respectively, the β_2 subunit was below the detection limit at this time in both rodent models. In the rat and mouse embryo, the first myocardial contractions occur around days 10 and 8, respectively [Lyons et al., 1990], indicating the presence of functional Ca^{2+} channels at early stages of cardiac ontogeny. Two case scenarios can be discussed. The first is that, in immature cardiomyocytes, the α_{1C} subunit alone constitutes a significant Ca^{2+} influx route. The second possibility is that transiently expressed fetal β subunit isoforms control α_{1C} function. Although heterologous expression studies have documented that the α_{1C} subunit can by itself form functional Ca^{2+} channels [reviewed in Perez-Reyes and Schneider, 1994], recent studies have suggested that all four β isoforms play an important structural role in the formation of stable α_{1C}-β complexes and their incorporation into the plasma membrane [Shistik et al., 1995; Gerster et al., 1999; Gao et al., 1999]. We therefore elucidated the presence of fetal cardiac β isoforms that could functionally substitute for the β_2 subunit isoform.

Fetal β Subunit Isoforms

In cardiac tissue of 14-day-old rat fetuses, four different cDNA (designated 4a, 4d1, 4d2, 4i) were amplified by RT-PCR with high homology to rat brain Ca^{2+} channel β_4 cDNA (Cchb4) [Castellano et al., 1993]. Since the N-terminal β_4 region could not be amplified from cardiac tissue, we conclude that cardiac and brain β_4 mRNA diverged in the N-terminal portion. However, starting with the first conserved domain of β subunits (DVSLEEDREA motif) (Fig. 4), the deduced amino acid sequence for the rat heart β_{4a} transcript shared complete identity to brain β_4 subunit, including the β_4-specific C-terminal domain. Two fetal cardiac β_4 transcripts (4d1, 4d2) lack the central 20-bp exon coding for the amino acid sequence AKQKQK. This exon is identical in β_1–β_4 and is subject to alternative splicing [Castellano and Perez-Reyes, 1994]. Exon skipping reported for renal β_4 [Yu et al., 1995] leads to a shift in the open reading frame and premature termination. Although both cDNAs (4d1, 4d2) predict truncated β_4 proteins, it is interesting to note, that the position of the second 59-nucleotide deletion, we observed in the 4d2 variant, corresponds to exon 8 in β_3 [Murakami et al., 1996] and might also be subject to alternative splicing. On the basis of these results, we conclude that developing cardiomyocytes express alternatively spliced isoforms arising from the β_4 gene.

Transient expression of the β_4 gene as well as alternative splicing of β_4 transcripts are supported by the detection of β_4 immunoreactive proteins in fetal rat hearts. Although the migration of the β_4-stained proteins predicts an unusual high molecular mass, these data strengthen our hypothesis that fetal β_4 isoforms are expressed to a reasonable level. Reciprocal expression profiles of β_4 and β_2 in rat

heart provide further evidence that β_4 may substitute for β_2 during fetal development.

Fetal α_{1C} Isoforms

A further interesting finding is the presence of two α_{1C} isoforms in fetal and neonatal hearts, but not in adult hearts. These results are consistent with data from Diebold et al. [1992], who showed that fetal and neonatal hearts express two different α_{1C} transcripts (designated S3A, S3B), whereas only the S3B form predominates in the adult rat heart. This developmental variability in cardiac isoforms arises from mutually exclusive splicing of two adjacent exons in the third membrane spanning region of the fourth domain. The alternative use of equal-sized exons [Diebold et al., 1992] should, however, not induce remarkable changes in the M_r of encoded α_{1C} proteins. Thus, the detection of two α_{1C}-stained bands migrating at 250 and 220 kDa in immature rodent hearts cannot be explained by the expression of both S3A and S3B transcripts in these preparations. It remains to be elucidated whether α_{1C} proteins encoded by either S3A or S3B display abnormal electrophoretic mobility or differ in their posttranslational processing, or both.

Since the expression of α_{1C} is not restricted to cardiac myocytes [Perez-Reyes and Schneider, 1994], the 220-kDa α_{1C} form of immature hearts may alternatively originate from smooth muscle cells, fibroblasts, or neuronal cells present in cardiac samples. However, previous results argue against this possibility. As in the neonatal heart, we identified two α_{1C} isoforms in the clonal cardiac cell line H9c2 [Hoch et al., 1998] derived from embryonic rat ventricular cells. These data suggest that fetal cardiomyocytes express an unique α_{1C} isoform in addition to the 250-kDa full-length α_{1C}. We speculate, that the transiently expressed fetal α_{1C} is functionally related to the DHP resistant Ca^{2+} channel identified in cardiomyocytes of 18-day-old fetal rats. This type of Ca^{2+} current decreased in amplitude and became less prominent during postnatal development [Tohse et al., 1992].

Maturation of Cardiac Ca^{2+} Channels

In this study, the highest relative expression levels for both α_{1C} and β_2 subunits were observed in newborn hearts. As development proceeds, α_{1C} protein levels decreased, while β_2 expression persisted. These results are surprising, as many previous reports on DHP binding sites and Ca^{2+} inward currents in postnatally developing hearts suggested the maturational increase in the expression of L-type channels [Renaud et al., 1989; Navaratnam and Khatter, 1991; Wibo et al., 1991; Wetzel et al., 1993; Huynh et al., 1992]. However, results and calculations from Vornanen [1996, and references therein] on the developmental changes of both the Ca^{2+} inward current and the sarcolemmal surface area clearly showed that the Ca^{2+} current density remains largely unaltered in rat cardiomyocytes between birth and adulthood, although the current amplitude increases strongly. Our data on unaltered β_2 subunit expression throughout postnatal development are concordant with these results [Vornanen, 1996] and suggest that the β_2 subunit plays a crucial role in the acquisition of sarcolemmal Ca^{2+} channels during cardiac maturation.

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