0031-3998/11/6906-0479
PEDIATRIC RESEARCH

Vol. 69, No. 6, 2011
Printed in U.S.A.

Perinatal and Postnatal Expression of Cav1.3 α_{1D} Ca^{2+} Channel in the Rat Heart

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ABSTRACT: The novel Cav1.3 (α_{1D}) L-type Ca^{2+} channel plays a significant role in sinoatrial (SA) and atrioventricular (AV) nodes function and in atrial fibrillation. However, the characterization of α_{1D} Ca^{2+} channel during heart development is very limited. We used real-time RT-PCR, Western blotting, and indirect immunostaining to characterize the developmental expression and localization of α_{1D} Ca^{2+} channel in rat hearts. Both protein and mRNA levels of α_{1D} Ca^{2+} channel decreased postnatally. Two forms of α_{1D} Ca^{2+} channel protein (250 and 190 kD) were observed, with the full-length (250 kD) channel protein being predominant in the prenatal stages. Both Western blots and confocal imaging demonstrated that α_{1D} Ca^{2+} channel protein was expressed in both atria and ventricles at fetal and neonatal stages but was absent in the adult ventricles. Interestingly, α_{1D} Ca^{2+} channel was also found at the nucleus/perinucleus of immature but not adult atrial cells. Furthermore, the nuclear staining was reproduced in adult atrial cell line, HL-1 cells, which possess immature properties. The data are first to show that α_{1D} Ca^{2+} channel has unique age-dependent expression profile and subcellular localization in the heart, suggesting a developmental stage-dependent specific function. **(*Pediatr Res* 69: 479–484, 2011)**

The L-type Ca^{2+} channel is a heterologomeric complex of α_1, β, and α_2/δ subunits (1). Four genes encode L-type Ca^{2+} channel α_1 subunits in mammals (α_{1C}, α_{1S}, α_{1D}, and α_{1F}) (1,2). α_{1C} (Cav1.2) represents the most abundant isoform in the cardiovascular system, whereas α_{1D} (Cav1.3) is expressed in neurons and neuroendocrine cells (3,4). It is therefore believed that the contribution of L-type Ca^{2+} current ($I_{Ca\text{-}L}$) to the physiology/pathophysiology of the heart is mainly mediated through α_{1C} Ca^{2+} channel.

The functional role of α_{1D} in the heart has been addressed by several published reports (5–7), including from our laboratory (8). The emerging consensus is that because of the low activation voltage (−60 to −50 mV) and abundant expression in the sinoatrial (SA) and atrioventricular (AV) nodes, α_{1D} plays an important role in the pacemaker activity and action potential conduction at the AV node. This is further supported by data showing that genetic deletion of α_{1D} causes sinus bradycardia and various degrees of AV block (5–7) and makes this mouse prone to atrial fibrillation (8). Compared with α_{1C}, the α_{1D} Ca^{2+} channel has less sensitivity to dihydropyridines (1,9,10). Despite these subtle differences, to date, there are no available pharmacological or biophysical approaches to functionally dissect α_{1D} from α_{1C} Ca^{2+} current in the native tissue.

Developmental change of the α_{1C} Ca^{2+} channel in the heart has been extensively studied (11,12). Lower α_{1C} Ca^{2+} channel expression levels have been demonstrated in immature hearts, followed by an increase with cardiac maturation in both rat and human (11,13). It is generally believed that a higher expression of the T-type Ca^{2+} channel and the Na/Ca exchanger compensate for the low levels of the α_{1C} Ca^{2+} channel and enable the immature heart to maintain functional contractility. However, there is very limited information on the developmental changes of the α_{1D} Ca^{2+} channel in the heart. This study is the first to characterize the expression levels of the α_{1D} Ca^{2+} channel and its subcellular localization during development in the rat heart.

METHODS

Use of rats. Fetal (17- to 20-d gestation), neonate (1- to 3-d old), juvenile (4- to 6-wk-old), and adult (6- to 8-mo-old) Sprague-Dawley rats of either sex used in this study were approved by IACUC at VA New York Harbor Healthcare System.

Real-time RT-PCR. TaqMan real-time RT-PCR was performed using 18S ribosomal RNA as internal control. Total RNAs were prepared from the atria at various developmental stages as previously described (14,15). Predesigned and labeled primer/Taqman probe sets for α_{1D} were purchased (Applied Biosystems, CA). The conditions for real-time RT-PCR was preheating at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles of shuttle heating at 95°C for 15 s and at 60°C for 1 min. The cycle threshold Ct value for each sample that was proportional to the log of the initial amount of input cDNA was calculated and plotted. 18S rRNA was used as internal control.

Protein extraction and Western blot. Membrane proteins were prepared as previously described (15). Same amount of membrane proteins (50–100 μg) were loaded for each lane of 4–12% SDS polyacrylamide gels. The immunoblots were incubated overnight at 4°C with 1:200 primary anti-α_{1D} Ca^{2+} channel antibodies (Calbiochem, CA), developed with horseradish peroxidase-labeled anti-rabbit antibody, and detected by enhanced chemiluminescence (Amersham, NJ). The density of protein bands was quantified by the National Institutes of Health image software (http://rsbweb.nih.gov/nih-image/). Anti-α_{1D} antibody preincubated with its antigenic peptide was included as a negative control.

Isolation of cardiac myocytes. Cardiac myocytes of juvenile and adult rats were obtained from Langendorff-perfused hearts as previously described (16). Hearts were perfused at 37°C with a HEPES-buffered solution containing 1.5 mg/mL collagenase type B (Boehringer Mannheim, Germany) for 8–15 min and then dispersed then dispersed in a KB solution containing (mmol/L): K

Received August 26, 2010; accepted December 29, 2010.

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Supported by National Institutes of Health [R01 HL-077494], Veterans Affairs MERIT grants [to M.B.], and Veteran Affairs MREP grant [to Y.Q.].

Abbreviations: $I_{Ca\text{-}L}$, L-type Ca^{2+} current

glutamate 70, KCl 30, KH_2PO_4 10, $MgCl_2$ 1, taurine 20, glucose 10, and HEPES 10. Isolation of fetal and neonatal rat atrial and ventricular myocytes was performed using chopping method with trypsin digestion as described (17), and cells were cultured on cover slips with Dulbecco's Modified Eagle's Medium containing 10% calf serum (GIBCO, CA), 2% penicillin/streptomycin, before use for immunostainings and transfections.

Maintenance of adult atrial HL-1 cells. The HL-1 cells were cultured in Claycomb medium supplemented with 10% fetal bovine serum (Invitrogen, CA), 2 mM L-glutamine, 100 μM norepinephrine, 100 U/mL penicillin, and 100 μg/mL streptomycin on precoated flasks with fibronectin (18).

Indirect immunofluorescence staining. Briefly, immunofluorescent stainings were performed as previously described (14). Cardiac cells were permeabilized, blocked, incubated overnight at 4°C with primary anti-α_{1D} antibodies against a peptide [(KY)DNKVTIDDYQEEADKD)] corresponding to residues 809–825 of rat brain α_{1D} subunit (1:200; Calbiochem, CA, and Sigma Chemical Co., MO), and detected with FITC-conjugated anti-rabbit antibody (1:200; Johnson Immunol, PA) and viewed with a confocal scanning laser microscope (MRC-600; Bio-Rad, CA) using XYZ scan. Surface plot was used to illustrate anti-α_{1D} antibody staining. Secondary antibody alone and anti-α_{1D} antibody preincubated with its antigenic peptide were included as negative controls.

Statistic analysis. Statistical comparisons were evaluated using unpaired student *t* test and one-way ANOVA as appropriate. Data are presented as mean ± SEM. A value of $p < 0.05$ is considered significant.

RESULTS

***Perinatal and postnatal expression level of α_{1D}* mRNA.** The α_{1D} Ca^{2+} channel mRNA level during heart development was carried out by real-time RT-PCR using total RNA isolated from fetal, neonatal, 4- to 6-wk-old, and 6- to 8-mo-old rat atrial tissue using 18S rRNA as an internal control. For easy comparison, the value of the α_{1D} Ca^{2+} channel mRNA at different developmental stages was normalized to that of the fetal stage. α_{1D} Ca^{2+} channel mRNA level decreased to 0.70 at neonatal stage, decreased to 0.36 at 4 wk, and reached the lowest level of 0.22 ($n = 3$, $p < 0.05$) at the adult stage. The results from three different experiments are summarized in Figure 1*A*.

Perinatal and postnatal expression of α_{1D} Ca^{2+} channel protein. The α_{1D} Ca^{2+} channel protein level at various stages was assessed by Western blot and shown in Figure 1*B*. At the fetal stage (lane 1), a full size (250 kD) and a short form (190 kD) of the α_{1D} Ca^{2+} channel protein were observed. At neonatal stage (lane 2), 6-wk-old (lane 3), and 6-mo-old (lane 5) atria, only the short form band was observed. However, none of the α_{1D} Ca^{2+} channel protein bands (250 and 190 kD) were observed in the 6-wk (lane 4) and 6-mo ventricles (lane 6). Both the full size and the short form bands were absent when preincubating the anti-α_{1D} antibody with its antigenic peptide (lane 7). The lower panel of Figure 1*B* shows a band corresponding to nonspecific proteins in each lane demonstrating that the absence of the α_{1D} protein in the adult stage is not due to a loading error. The α_{1D} Ca^{2+} channel protein level decreased postnatally and reached steady level at 6 wk. The value of α_{1D} Ca^{2+} channel protein at different developmental stages was normalized to that of the 6-wk and 6-mo-old atria. The α_{1D} Ca^{2+} channel protein level was 12 ± 2.5 and 4.5 ± 0.6 fold higher at fetal and neonatal stage, respectively, ($n = 3$, $p < 0.05$).

Developmental-dependent subcellular localization of the α_{1D} Ca^{2+} channel. Subcellular localization of the α_{1D} Ca^{2+} channel was carried out by confocal indirect immunostaining

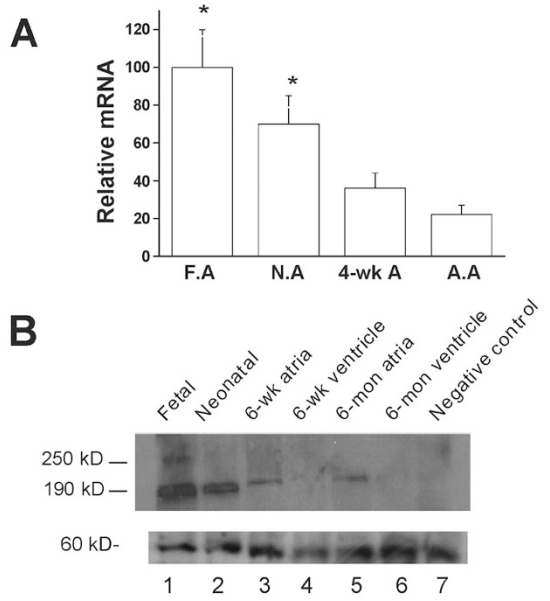

Figure 1. α_{1D} Ca^{2+} channel mRNA and proteins levels during heart development using real-time PCR and Western blot, respectively. (*A*) α_{1D} Ca^{2+} channel mRNA during rat heart development by real-time RT-PCR using 18S rRNA as internal control. Value of the fetal stage was set at 100 for easy comparison. * indicates $p < 0.05$ compared with adult stage. F.A: fetal atria; N.A: neonatal atria; A.A: adult atria. (*B*) Representative Western blot experiment using anti-α_{1D} Ca^{2+} channel antibody. Total membrane protein (100 μg) was loaded in each lane. Lane 1, fetal stage; lane 2, neonatal stage; lane 3, 6-wk atria; lane 4, 6-wk ventricles; lane 5, 6-mo atria; lane 6, 6-mo ventricle; and lane 7, negative control (anti-α_{1D} Ca^{2+} channel antibody preincubated with antigenic peptide using membrane protein from fetal stage). (*B*) *Lower part*: nonspecific protein bands observed in each lane, indicating that absence of α_{1D} protein in the adult stage is not secondary to loading error.

using the anti-α_{1D} antibody. Sarcolemmal staining was observed in the fetal (Fig. 2*A*), neonatal (Fig. 2*B*), 6-wk (Fig. 2*C* and *D*), and 6-mo (Fig. 2*E*) atrial cells. Ventricular cells at both the fetal (Fig. 3*A*) and neonatal (Fig. 3*B*) stages showed similar staining pattern to atrial cells (Fig. 2*A* and *B*), indicating that the α_{1D} Ca^{2+} channel protein is expressed in both the atria and the ventricles of the immature hearts. Interestingly, no staining was observed in the 6-wk and 8-mo-old ventricular cells (Fig. 3*C* and *D*). The staining pattern of the α_{1D} Ca^{2+} channel in the atrial cells was not observed when using the secondary antibody alone (data not shown) or using the anti-α_{1D} antibody preincubated with its antigenic peptide at the fetal (Fig. 4*A*), the neonatal (Fig. 4*B*), 6-wk (Fig. 4*C*), and 6-mo (Fig. 4*D*) stages, indicating the specificity of the anti-α_{1D} antibody.

Interestingly, in addition to the sarcolemmal staining, a unique nuclear staining was observed in immature hearts (Figs. 2*A* and *B* and 3*A* and *B*). This staining pattern was not seen in 6-wk and 6-mo-old rat atrial cells (Fig. 2*C–E*). The specificity of the nuclear staining was demonstrated by the use of anti-α_{1D} antibodies from two commercial vendors that showed the same staining pattern (data not shown). In addition, the nuclear staining was not observed in the negative control experiments in which the anti-α_{1D} antibody was pre-

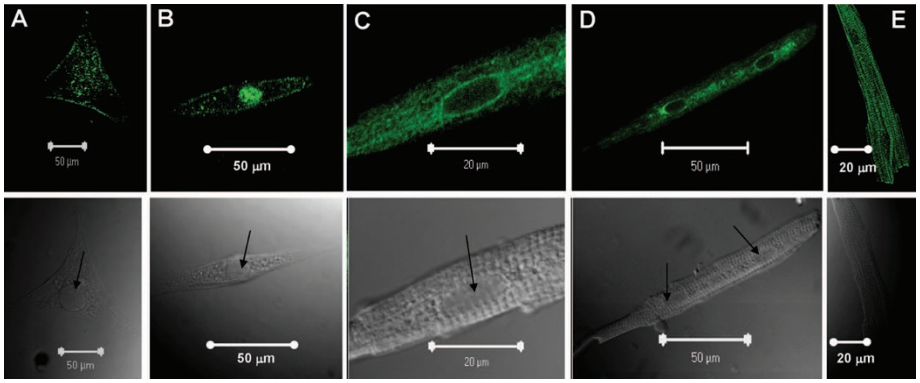

Figure 2. Representative confocal images of isolated atrial myocytes using anti-α_{1D} Ca^{2+} channel antibody. The *upper panels* show the staining pattern of the α_{1D} Ca^{2+} channel protein using FITC-labeled secondary antibody. The *lower panels* show the phase controls of the cells at different developmental stages. *A*, fetal; *B*, neonatal; *C*, 6 wk (amplified from *D*); *D*, 6 wk; *E*, 6 mo. *Arrows* indicate the nucleus.

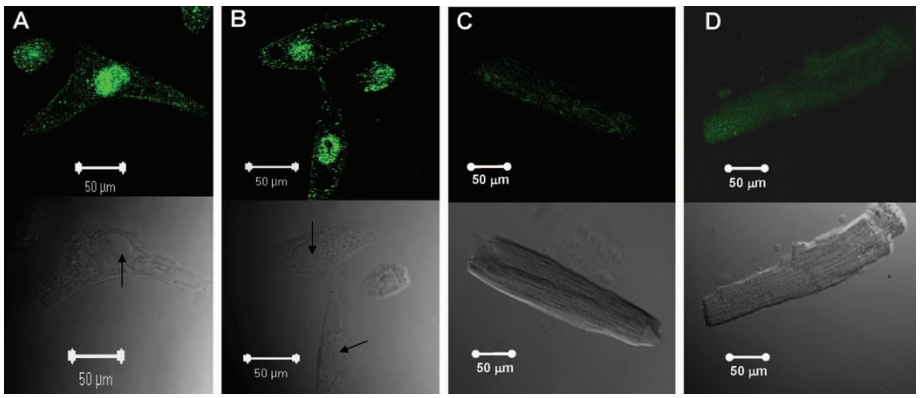

Figure 3. Representative confocal images using anti-α_{1D} Ca^{2+} channel antibody on ventricular cells from various developmental stages: *A*, fetal ventricular cells; *B*, neonatal ventricular cells; *C*, 6-wk ventricular cell; *D*, 8-mo ventricular cell.

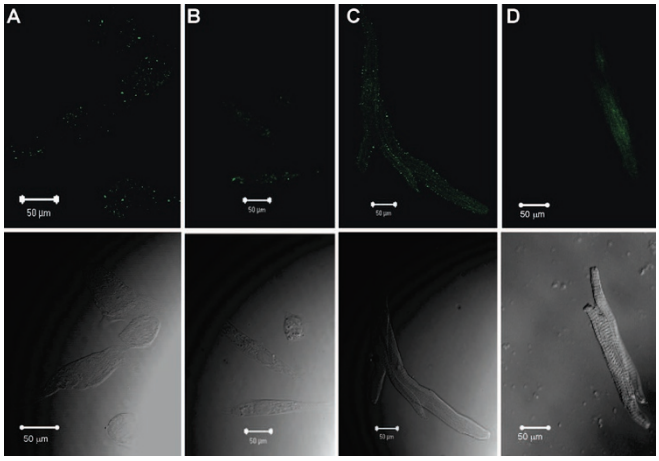

Figure 4. Representative confocal images of negative controls using anti-α_{1D} Ca^{2+} channel antibody preincubated with its antigenic peptide: *A*, fetal atrial cells; *B*, neonatal atrial cells; *C*, 6-wk atrial cells; *D*, 6-mo atrial cell. *Arrows* indicate the nucleus.

incubated with its antigenic peptide (Fig. 4*A* and *B*) and with the secondary antibody alone (data not shown). Immunostaining intensity across the cells was assessed by surface plot and shows that the α_{1D} protein is present at the sarcolemma but is more prominent at the nuclear region of fetal and neonatal stages (Fig. 5*A* and *B*), compared with adult stages (Fig. 5*C* and *D*).

Expression of the α_{1D} Ca^{2+} channel in adult atrial HL-1 cells. To confirm that the nuclear staining was unique only to the immature cells, we took advantage of a tumor-derived mouse atrial cell line, HL-1 cells, to perform immunostaining using the anti-α_{1D} antibody. The unique feature of the HL-1 cells is that they retain the properties of the normal adult atrial cells, but ultrastructurally resemble immature mitotic mouse atrial myocytes. The expression of α_{1D} Ca^{2+} channel in HL-1 cells was demonstrated by RT-PCR using the α_{1D} specific primer (Fig. 6*A*) and by Western blot (Fig. 6*B*) using the anti-α_{1D} antibody. Interestingly, in these HL-1 cells, the α_{1D} Ca^{2+} channel was found to be on the sarcolemma and also in the nucleus, a staining pattern that is indistinguishable from that of the fetal and neonatal cardiac myocytes (Fig. 6*C*), suggesting that the α_{1D} Ca^{2+} channel might play unique roles in the immature cardiac cells.

DISCUSSION

Although the developmental expression profile of the α_{1C} Ca^{2+} channel has been extensively studied previously (11,13,19), to date, no such information is available for the α_{1D} Ca^{2+} channel. This study is the first to characterize the expression and subcellular localization of α_{1D} Ca^{2+} channel during heart development. The striking findings of this study are the abundant level, the universal expression, the unique nuclear localization, and the existence of the full-length (250 kD) α_{1D} Ca^{2+} channel in the immature heart. In contrast, only the short form (190 kD) of the α_{1D} Ca^{2+} channel was expressed in the adult stage and its expression is restricted to the atria but not to the ventricles. Interestingly, the nuclear localization of the α_{1D} Ca^{2+} channel was not observed in the adult atrial cells.

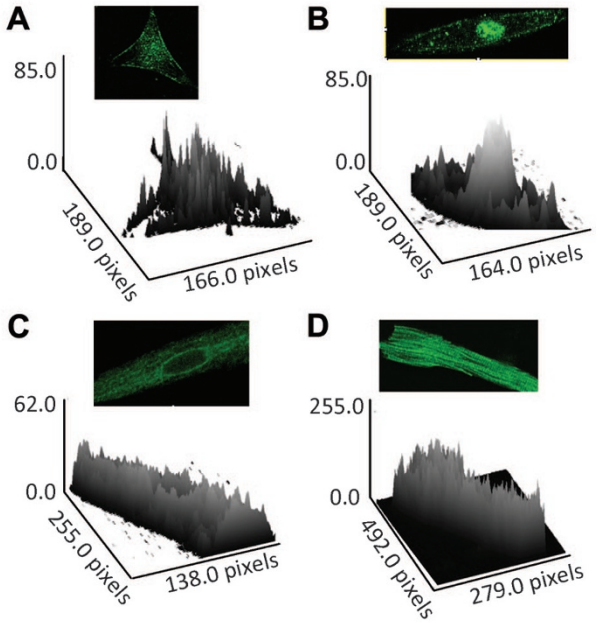

Figure 5. Surface plots corresponding to Figure 2 showing staining intensity of the α_{1D} protein across the atrial cells including sarcolemma and perinuclear regions at different developmental stages. *A*, fetal; *B*, neonatal; *C*, 6-weeks; *D*, 6-months. Note prominent nuclear staining at both fetal and neonatal stages compared with adult stages.

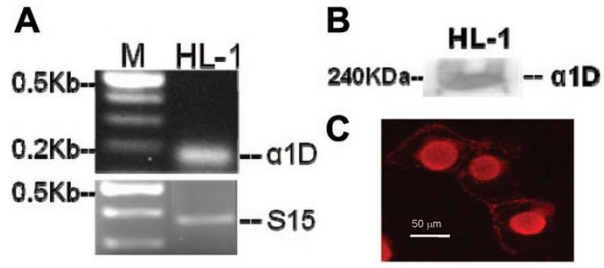

Figure 6. (*A*) Expression and localization of α_{1D} Ca channel in HL-cells: Transcript of 180 bp corresponding to the α_{1D} Ca^{2+} channel was amplified from HL-1 cells. A 361-bp housekeeping gene S15 confirms accuracy of cDNA integrity and gel loading techniques. M indicates markers. (*B*) Expression of the α_{1D} Ca^{2+} channel protein in HL-1 cells by Western blot using anti-α_{1D} Ca^{2+} channel antibody. (*C*) Confocal immunostaining of the α_{1D} Ca^{2+} channels in HL-1 cells using anti-α_{1D} Ca^{2+} channel antibody.

Altogether, the data suggest that the α_{1D} Ca^{2+} channel may play unique roles in the immature heart. However, because of the unavailable pharmacological or biophysical approaches to separate the α_{1D} from the α_{1C} channel currents, the functional relevance of the α_{1D} Ca^{2+} channel during heart development was not sought.

Distinct subcellular localization of the α_{1D} Ca^{2+} channel at different developmental stages. An intriguing observation of this study is the age-dependent subcellular localization of the α_{1D} Ca^{2+} channel. While in the fetal and neonatal stages, the α_{1D} Ca^{2+} channel is highly expressed in the nucleus in addition to the sarcolemma, it gradually moves to the perinuclear region and eventually resolves from the perinuclear areas at the adult stage. The specificity of the nuclear/perinuclear staining was confirmed with different experimental approaches. First, the anti-α_{1D} Ca^{2+} channel antibodies from two commercial resources yielded the same staining patterns. Second, the nuclear staining was not observed when the anti-α_{1D} antibody was preincubated with its antigenic peptide. Third, using the same antibody, the nuclear staining was observed in only the fetal and the neonatal stages but not in the adult stage. Fourth, the α_{1D} Ca^{2+} channel was observed in the nucleus of adult atrial HL-1 cells, which possess properties of the immature cardiac cells. Finally, consistent with the present results, Zhang *et al.* (7), using the same anti-α_{1D} antibody (Calbiochem, San Diego, CA), showed similar staining pattern for the adult atrial cells, in which the nuclear staining was not observed. Furthermore, this anti-α_{1D} antibody did not show any specific staining in atrial cells from the α_{1D} Ca^{2+} channel knockout mice. We have also ruled out the possibility that the observed nuclear staining may be an artifact associated with the cultures of fetal and neonatal atrial and ventricular cells because similar staining patterns of the α_{1D} Ca^{2+} channel were also observed in freshly isolated human fetal cardiac cells (15). Altogether, these data demonstrate that the nuclear staining in fetal and neonatal cardiac myocytes is specific. Further support for this nuclear/perinuclear localization of the α_{1D} Ca^{2+} channel is that the expression of ion channels in the nucleus has been reported for Cl channels, Ca-ATPases, $InsP_3$ receptors, and K channels (20–24). The exact role of the α_{1D} Ca^{2+} channel in the nucleus is not yet known and warrant further investigations.

It is also noteworthy that the α_{1D} Ca^{2+} channel was not expressed in the adult rat ventricles. This observation is supported by studies from Takemura *et al.* (25), who showed similar result, and by Zhang *et al.* (7), who showed a significantly lesser expression levels of the α_{1D} Ca^{2+} channel in the adult mice ventricles. This developmental expression pattern for the α_{1D} Ca^{2+} channel is strikingly similar to that of the T-type Ca^{2+} channel (11) with the exception that the T-type Ca^{2+} channel is reexpressed in the adult ventricles under pathological conditions (26,27). Whether the α_{1D} Ca^{2+} channel is reexpressed in the adult ventricles under pathological conditions remains to be determined.

Expression levels of the α_{1D} Ca^{2+} channel in the heart at different stages. Studies involving with developmental changes have faced the challenge of choosing an internal control with invariant expression. Among the commonly used internal control housekeeping genes, such as glyceraldehydes phosphate dehydrogenase (GAPDH), β-actin, and ribosomal RNA (18S and 28S), 18S rRNA seems to be the better standard as it shows constant levels throughout gestation and its expression does not appear to vary between individuals (28,29). On the other hand, the expression level of GAPDH and β-actin varies across tissues during cell proliferation (28,30). Significant variation in GAPDH expression has also been reported between individuals and during development (28,29,31). In this study, α_{1D} Ca^{2+} channel mRNA level, determined by real-time RT-PCR using 18S rRNA as an internal control, decreased after birth. Postnatal decrease of the α_{1D} Ca^{2+} channel mRNA was paralleled by the protein level determined

by Western blot. Physiologically, the more abundant expression of the α_{1D} Ca^{2+} channel in the fetal cardiac stage is likely beneficial because of the low levels of the α_{1C} Ca^{2+} channel expression (19,32) and for the less abundant sarcoplasmic reticulum in the fetal heart compared with the adult heart (33,34).

Despite the abundant expression of the α_{1D} Ca^{2+} channel in the fetal stage, the knockout of the α_{1C} Ca^{2+} channel in mice is embryonic lethal at d 14.5 postcoitum (35), while the $\alpha_{1D}^{-/-}$ mice survives (5,7,36). This suggests that the α_{1D} Ca^{2+} channel in the immature heart may be important to cellular functions other than excitation-coupling. Indeed, the unique nuclear localization of the α_{1D} channel in the fetal and neonatal stages also points to other unique functions of the α_{1D} in the immature hearts.

Two forms of the α_{1D} Ca^{2+} channel are present in the immature hearts. Two forms of the α_{1D} Ca^{2+} channel protein, a full length (250 kD) and a short form (190 kD) were observed in the immature heart. A splicing site at the C-terminal region of α_{1D} has been reported (37,38). A 58-bp deletion results in a frame shift that produces a premature termination codon giving rise to the α_{1D} subunit that differs in length by 535 amino acid, with a corresponding molecular weight of 190 kD (37,38). On the other hand, experiments in the cardiac myocytes and neurons indicate that the C terminus of the α_{1C} channel is proteolytically cleaved, yielding a truncated channel (39,40), with the full length observed in only the fetal heart (13,41), indicating an increased proteolytic activity in the adult heart. The authors also reported a novel mechanism of the α_{1C} channel-associated transcription regulation in which the proteolytic C-terminal fragment of the α_{1C} Ca^{2+} channel translocates to the nucleus and regulates gene transcriptions (41). It is unclear whether these two sizes of α_{1D} protein observed in this study are the products of proteolytic activity or splicing variant. Whether these spliced variants are developmentally regulated and whether the observed differential localization of the protein is related to the expression of different splice variants are unknown and warrant further investigations.

In conclusion, the α_{1D} Ca^{2+} channel is a newly discovered L-type Ca^{2+} channel isoform in the heart. It has distinct age-dependent expression and subcellular localization, suggesting unique function during heart development.

Acknowledgments. We thank Dr. Seino and Dr. Striessnig for providing the plasmids used in this study.

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