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Perinatal and Postnatal Expression of Cav1.3 α_{1D} Ca²⁺ Channel in the Rat Heart

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Abstract

The novel Cav1.3 (α_{1D}) L-type Ca²⁺ channel plays a significant role in sino-atrial, atrioventricular nodes function and in atrial fibrillation. However, the characterization of α_{1D} Ca²⁺ 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} Ca²⁺ channel in rat hearts. Both protein and mRNA levels of α_{1D} Ca²⁺ channel decreased postnatally. Two forms of α_{1D} Ca²⁺ channel protein (250 kD and 190 kD) were observed, with the full length (250kD) channel protein being predominant in the prenatal stages. Both Western blots and confocal imaging demonstrated that α_{1D} Ca²⁺ channel protein was expressed in both atria and ventricles at fetal and neonatal stages but was absent in the adult ventricles. Interestingly, α_{1D} Ca²⁺ 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} Ca²⁺ channel has unique age-dependent expression profile and subcellular localization in the heart, suggesting a developmental stage dependent specific function.

INTRODUCTION

The L-type Ca²⁺ channel is a heterologomeric complex of α_1 , β , and α_2/δ subunits (1). Four genes encode L-type Ca²⁺ channel α_1 subunits in mammals (α_{1C} , α_{1S} , α_{1D} , 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 Ca²⁺ current (I_{Ca-L}) to the physiology/pathophysiology of the heart is mainly mediated through α_{1C} Ca²⁺ channel.

The functional role of α_{1D} in the heart has been addressed by several published reports (5–7) including from our lab (8). The emerging consensus is that because of the low activation voltage (–60mV to –50mV) and abundant expression in the SA and AV nodes, α_{1D} plays an important role in the pacemaker activity (diastolic depolarization) and action potential

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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 the mouse prone to atrial fibrillation (8). Compared with α_{1C} , the α_{1D} Ca^{2+} 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 (17–20 days gestation), Neonate (1–3 day old), juvenile (4- to 6- weeks old) and adult (6- to 8-months 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 μg to 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 (ELC) (Amersham, NJ). The density of protein bands was quantified by the NIH *image* software (<http://rsbweb.nih.gov/nih-image/>). Anti- α_{1D} antibody pre-incubated 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 to 15 minutes and then dispersed then dispersed in a KB solution containing (mmol/L): K glutamate 70, KCl 30, KH_2PO_4 10, MgCl_2 1, taurine 20, glucose 10, 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 μ M norepinephrine, 100 U/ml penicillin and 100 μ g/ml streptomycin on pre-coated 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, 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 pre-incubated 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 \pm 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, 4–6 weeks and 6–8-months old rat atrial tissue using 18sRNA 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, to 0.36 at 4-week 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 Fig 1A.

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 Fig 1B. 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 6-week (lane 3) and 6-month (lane 5) old atria, only the short form band was observed. However, none of the α_{1D} Ca^{2+} channel protein bands (250 kD and 190 kD) were observed in the 6-week (lane 4) and 6-month ventricles (lane 6). Both the full size and the short form bands were absent when pre-incubating the anti- α_{1D} antibody with its antigenic peptide (lane 7). The lower panel of Fig 1B shows a band corresponding to non-specific 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-week. The value of α_{1D} Ca^{2+} channel protein at different developmental stages was normalized to that of the 6-week and 6-month 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 using the anti- α_{1D} antibody. Sarcolemmal staining was observed in the fetal (Fig 2A), neonatal (Fig 2B), 6-week (Fig 2C and D) and 6-month (Fig 2E) atrial cells. Ventricular cells at both the fetal (Fig 3A) and neonatal (Fig 3B) stages showed similar staining pattern to atrial cells (Fig 2A and 2B), 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-week and 8-month old ventricular cells (Fig 3C and 3D). 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 4A), the neonatal (Fig 4B), 6-week (Fig 4C) and 6-month (Fig 4D) 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 (Fig 2A and 2B, Fig 3A and 3B). This staining pattern was not seen in 6-week and 6-month old rat atrial cells (Fig 2C, 2D and 2E). The specificity of the nuclear staining was demonstrated by the use of anti- α_{1D} antibodies from two commercial vendors which 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 preincubated with its antigenic peptide (Fig 4A and 4B), 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 5A and 5B), compared to adult stages (Fig 5C and 5D).

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 6A) and by Western blot (Fig 6B) 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 6C) 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. 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. Lastly, consistent with the present results, Zhang et al., 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 (7). 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 since 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, InsP₃ 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.*, which showed similar result and (25) and by Zhang *et al.*, who showed a significantly lesser expression levels of the α_{1D} Ca^{2+} channel in the adult mice ventricles (7). 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 re-expressed in the adult ventricles under pathological conditions (26, 27). Whether the α_{1D} Ca^{2+} channel is re-expressed 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), 18sRNA appears 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 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 18sRNA 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 day 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 base pair 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 190KD (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 is 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.

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ABBREVIATIONS

| | |
|------------|--------------------------|
| I_{Ca-L} | L-type Ca^{2+} current |
| WT | Wild type |

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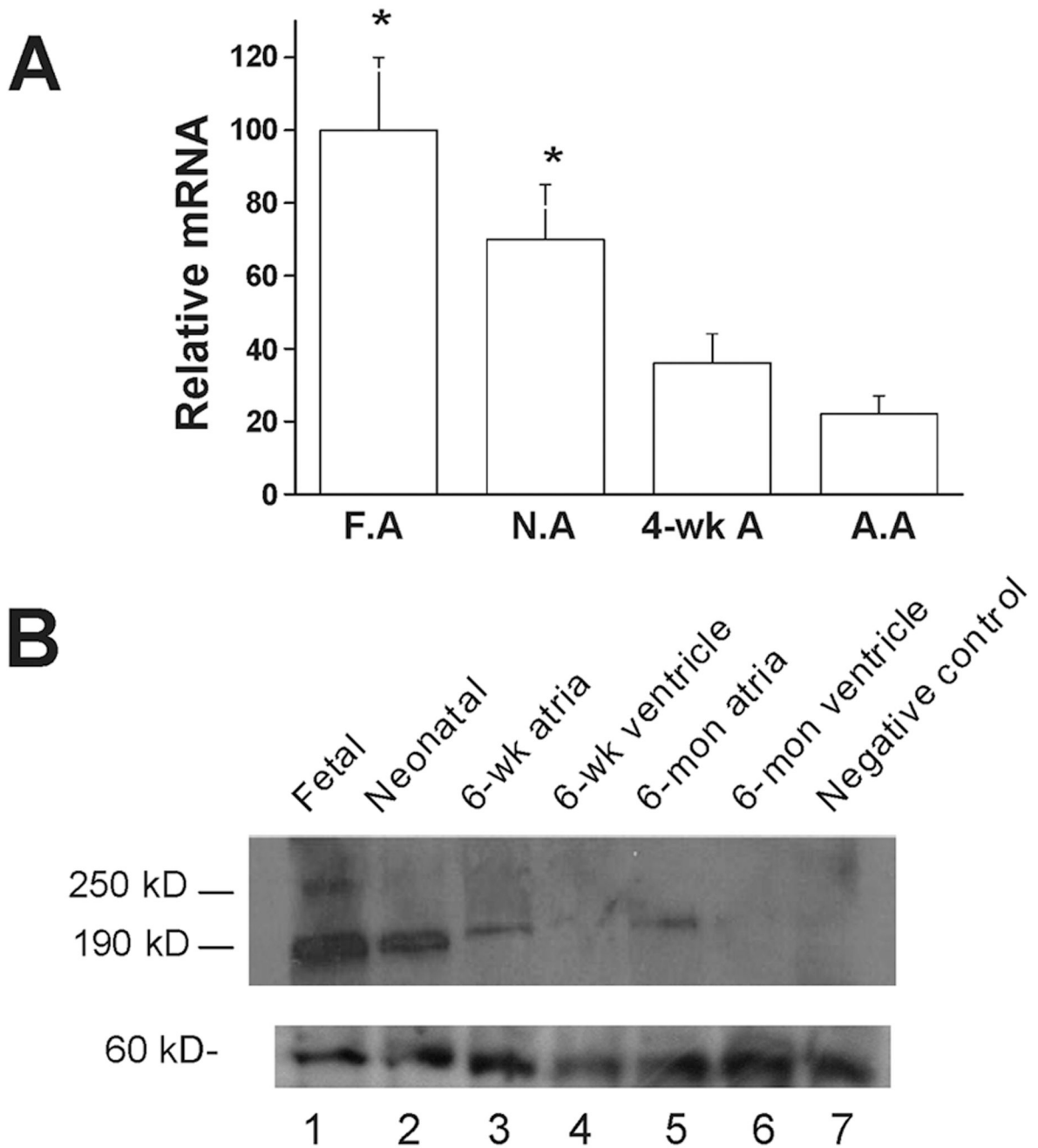


Figure 1. α_{1D} Ca²⁺ channel mRNA and proteins levels during heart development using real-time PCR and Western blot respectively. **A:** α_{1D} Ca²⁺ channel mRNA during rat heart development by real-time RT-PCR using 18sRNA as internal control. Value of the fetal stage was set at 100 for easy comparison. * indicates p<0.05 compared to adult stage. F.A: fetal atria; N.A: neonatal atria; A.A: adult atria. **B:** Representative Western Blot experiment using anti- α_{1D} Ca²⁺ channel antibody. Total membrane protein (100 μ g) was loaded in each lane. Lane 1, fetal stage; Lane 2, neonatal stage; lane 3, 6-weeks atria; lane 4, 6-week ventricles; lane 5, 6-months atria; lane 6, 6-months ventricle and lane 7, negative control (Anti- α_{1D} Ca²⁺ channel antibody preincubated with antigenic peptide using membrane protein from fetal stage).

Panel B, lower part, shows non-specific protein bands observed in each lane, indicating that absence of α_{1D} protein in the adult stage is not secondary to loading error.

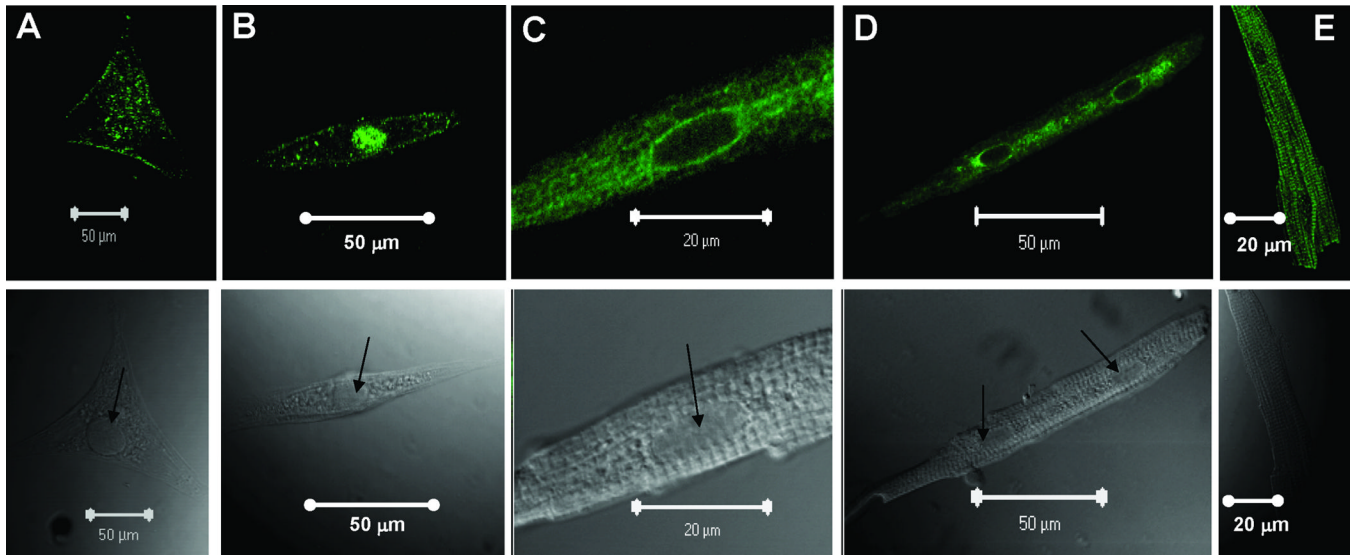


Figure 2.

Representative confocal images of isolated atrial myocytes using anti- α_{1D} Ca^{2+} channel antibody. 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-weeks (amplified from panel D), **D:** 6-weeks, **E:** 6-months. Arrows indicate the nucleus.

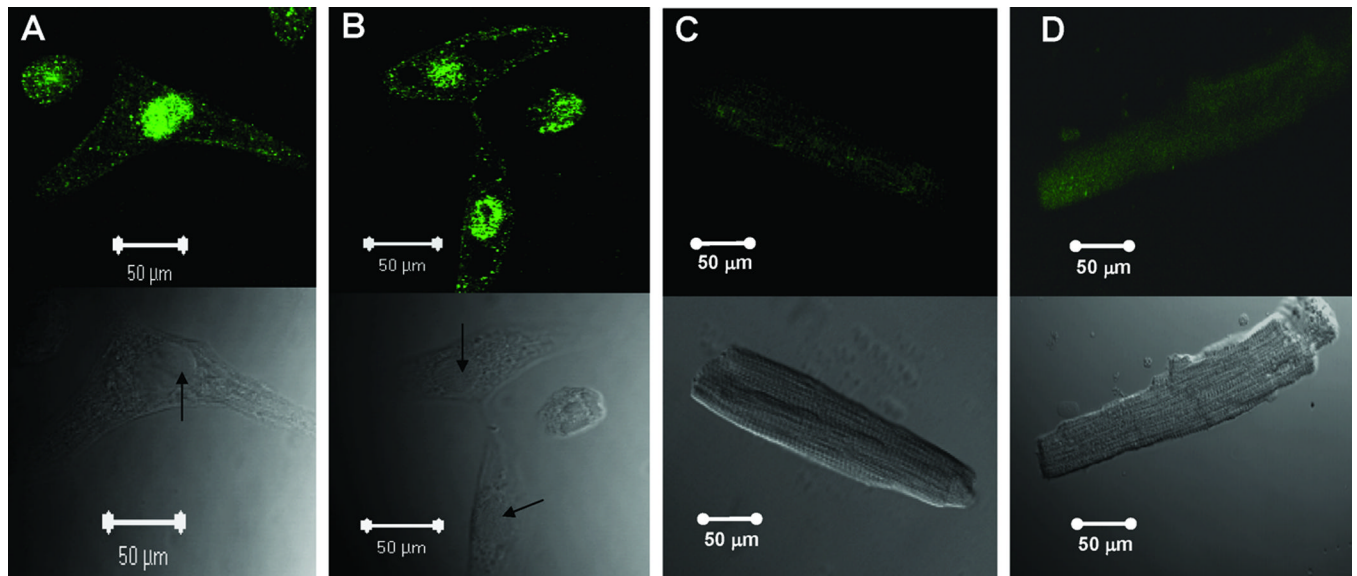


Figure 3. Representative confocal images using anti- α_{1D} Ca^{2+} channel antibody on ventricular cells from various developmental stages: **A:** fetal ventricular cells, **B:** neonatal ventricular cells, **C:** 6-weeks ventricular cell, **D:** 8-months ventricular cell.

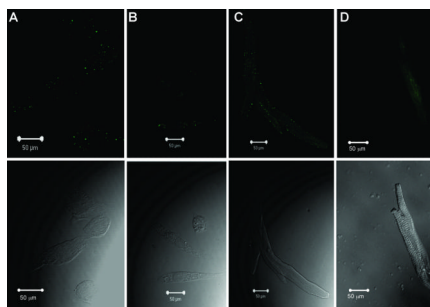


Figure 4. Representative confocal images of negative controls using anti- α_{1D} Ca^{2+} channel antibody preincubated with its antigenic peptide: **A:** fetal atrial cells, **B:** neonatal atrial cells, **C:** 6-weeks atrial cells, **D:** 6-months atrial cell. Arrows indicate the nucleus.

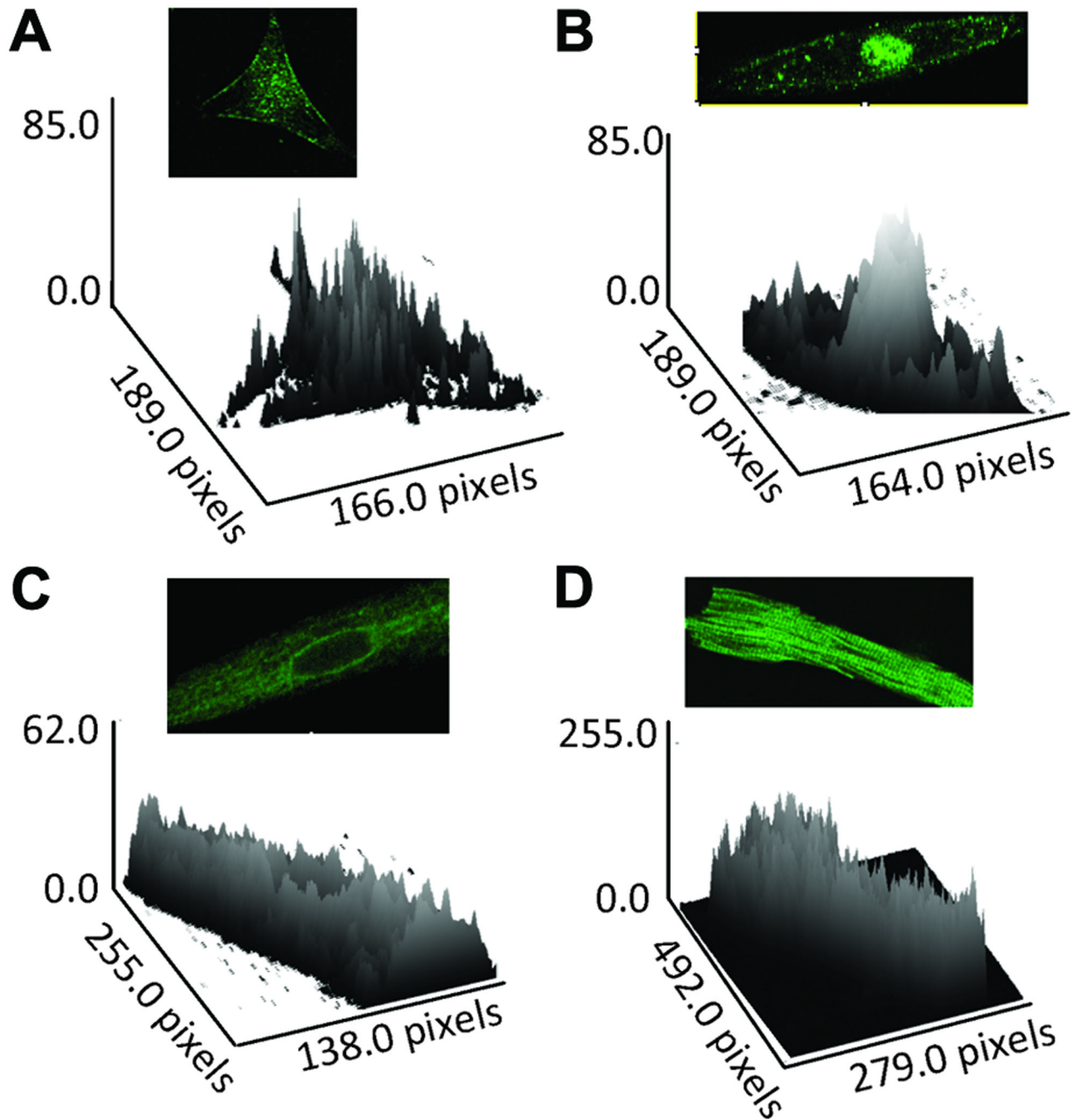


Figure 5.

Surface plots corresponding to Figure 2 showing staining intensity of the α_{1D} protein across the atrial cells including sarcolemma and nucleus at different developmental stages. **A:** fetal, **B:** neonatal, **C:** 6-weeks, **D:** 6-weeks, **E:** 6-months. Note prominent nuclear staining at both fetal and neonatal stages compared with adult stages. Note that nuclear staining is more prominent than sarcolemmal staining in the fetal and neonatal atria (panels A and B) compared to 6 weeks and 6 months old atria (panels D and E).

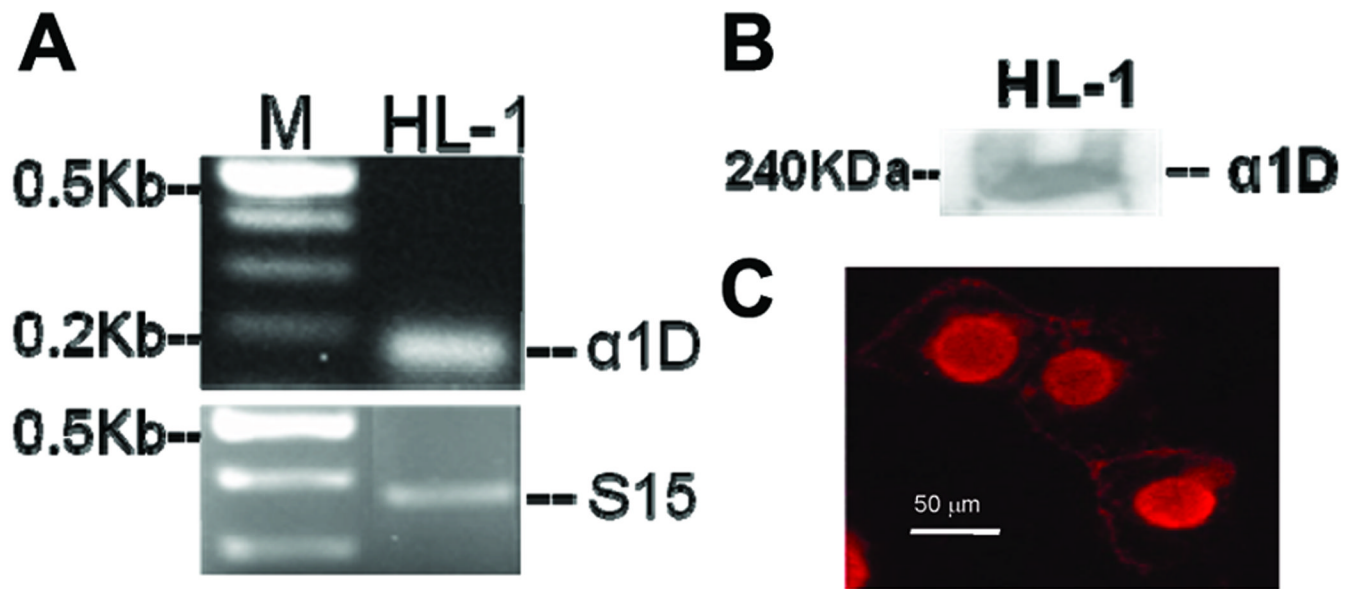


Figure 6. Expression and localization of α_{1D} Ca channel in HL-1 cells

A. Transcript of 180 bp corresponding to the α_{1D} Ca²⁺ 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} Ca²⁺ channel protein in HL-1 cells by Western blot using anti- α_{1D} Ca²⁺ channel antibody. **C:** Confocal immunostaining of the α_{1D} Ca²⁺ channels in HL-1 cells using anti- α_{1D} Ca²⁺ channel antibody.