Novel Molecular Mechanism Involving α_{1D} (Cav1.3) L-Type Calcium Channel in Autoimmune-Associated Sinus Bradycardia

Yongxia Qu, MD, PhD; Ghayath Baroudi, PhD; Yuankun Yue, MD; Mohamed Boutjdir, PhD

Background—Congenital heart block (CHB) is an autoimmune disease that affects fetuses/infants born to mothers with anti-Ro/La antibodies (positive IgG). Although the hallmark of CHB is complete atrioventricular block, sinus bradycardia has been reported recently in animal models of CHB. Interestingly, knockout of the neuroendocrine α_{1D} Ca channel in mice results in significant sinus bradycardia and atrioventricular block, a phenotype reminiscent to that seen in CHB. Here, we tested the hypothesis that the α_{1D} Ca channel is a novel target for positive IgG.

Methods and Results—Reverse transcription–polymerase chain reaction, confocal indirect immunostaining, and Western blot data established the expression of the α_{1D} Ca channel in the human fetal heart. The effect of positive IgG on α_{1D} Ca current (I_{Ca-L}) was characterized in heterologous expression systems (tsA201 cells and Xenopus oocytes) because of the unavailability of α_{1D}-specific modulators. α_{1D} I_{Ca-L} activated at negative potentials (between -60 and -50 mV). Positive IgG inhibited α_{1D} I_{Ca-L} in both expression systems. This inhibition was rescued by a Ca channel activator, Bay K8644. No effect on α_{1D} I_{Ca-L} was observed with negative IgG and denatured positive IgG. Western blot data showed that positive IgG binds directly to α_{1D} Ca channel protein.

Conclusions—The data are the first to demonstrate (1) expression of the α_{1D} Ca channel in human fetal heart, (2) inhibition of α_{1D} I_{Ca-L} by positive IgG, and (3) direct cross-reactivity of positive IgG with the α_{1D} Ca channel protein. Given that α_{1D} I_{Ca-L} activates at voltages within the pacemaker's diastolic depolarization, inhibition of α_{1D} I_{Ca-L} in part may account for autoimmune-associated sinus bradycardia. In addition, Bay K8644 rescue of α_{1D} I_{Ca-L} inhibition opens new directions in the development of pharmacotherapeutic approaches in the management of CHB. (***Circulation*. 2005;111:3034-3041.**)

Key Words: antibodies ■ sinoatrial node ■ heart block ■ ion channels ■ calcium

Autoimmune-associated congenital heart block (CHB) is a passively acquired autoimmune disease. Fetal injury is presumed to occur as a consequence of transplacental maternal autoantibodies against the intracellular ribonucleoproteins 48-kDa SSB/La, 52-kDa SSA/Ro, and 60-kDa SSA/Ro.[1] CHB historically is characterized by irreversible various degrees of AV block, not accompanied by any cardiac anatomic abnormalities.[1] Detection of CHB occurs after 16 weeks of gestation, when maternal antibodies effectively gain access to the fetal circulation.[2,3] The incidence of CHB is variable, ≈1 in 11 000 in the general population, but rises to 5 in 100 in patients with lupus.[2] CHB carries substantial morbidity and mortality approaching 30%, with >60% of affected children requiring lifelong pacemakers.[2] The establishment of both active (by injection of Ro/La antigens)[4] and passive (by injection of anti-Ro/La antibodies)[5] mouse models of CHB provides the most compelling evidence that maternal anti-Ro/La antibodies cause CHB.

Because AV block has been the hallmark phenotype for CHB, the AV node, rather than the sinoatrial (SA) node, has been the main focus of previous publications[4–6] and during clinical diagnosis of CHB.[2] In this regard, sinus bradycardia unrelated to AV block was first reported in animal models of CHB.[4,5] This was subsequently confirmed by Brucato et al[7] and Menon et al,[8] who reported significant sinus bradycardia in infants born to mothers seropositive to anti-Ro antibodies. The high incidence of sinus bradycardia in mouse models of CHB and in some affected infants indicates that the spectrum of ECG abnormalities in CHB extends beyond the AV node to affect the SA node. This previously underappreciated autoimmune-associated sinus bradycardia has drawn intense clinical and basic research attention because it may serve as a clinical marker for the detection of CHB, given that sinus bradycardia often precedes AV block.

In previous investigations of the mechanism of AV block in CHB, we demonstrated that positive IgG from mothers with CHB infants selectively inhibits α_{1C} L-type (I_{Ca-L}) and T-type (I_{Ca-T}) Ca current, but did not affect the pacemaker current I_f, I_k, and I_{Na}.[4,6,9–11] We proposed that inhibition of α_{1C} I_{Ca-L} could account for the AV block seen in CHB, because

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From the Molecular and Cellular Cardiology Program, VA New York Harbor Healthcare System (Y.Q., G.B., Y.Y., M.B.), SUNY Downstate Medical Center (Y.Q., G.B., M.B.), and NYU School of Medicine (M.B.), New York, NY.

Correspondence to Dr Mohamed Boutjdir, Research and Development Office (151), VA New York Harbor Healthcare System, 800 Poly Place, Brooklyn, NY 11209. E-mail mohamed.boutjdir@med.va.gov

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impulse conduction in the AV node depends critically on I_{Ca-L}. α_{1C} I_{Ca-L} activates at more positive (−40 and −30 mV) potentials, whereas SA node pacemaker depolarization occurs between −60 and −40 mV.[12] Thus, the contribution of α_{1C} I_{Ca-L} to diastolic depolarization of the SA node is generally considered to be minor. It is therefore logical to hypothesize that inhibition of α_{1C} I_{Ca-L} by positive IgG will not be the only factor contributing to sinus bradycardia reported in CHB. In support of this hypothesis are recent reports showing that the neuroendocrine α_{1D} Ca channel, which with the α_{1C} Ca channel contributes to the formation of I_{Ca-L}, is also expressed in the heart, specifically in the SA node and atria but not in adult ventricles.[13] α_{1D} I_{Ca-L} differs from α_{1C} I_{Ca-L} in that α_{1D} I_{Ca-L} activates at a more negative (−25mV) membrane potential. This unique biophysical property enables α_{1D} I_{Ca-L} to play a more important role in spontaneous depolarization in the SA node. In fact, α_{1D} Ca channel knockout mice exhibit profound sinus bradycardia.[14–16] These observations offer convincing evidence to support an important role for α_{1D} I_{Ca-L} in SA node pacemaking and evoke the possibility that the α_{1D} Ca channel may be a novel target for positive IgG and thus contribute to the development of autoimmune-associated sinus bradycardia. To establish that the α_{1D} Ca channel is a target for positive IgG, at least 2 criteria must be met: (1) the α_{1D} Ca channel should be expressed in human fetal heart, and (2) α_{1D} I_{Ca-L} should be inhibited by positive IgG. In the present study, we combined reverse transcription–polymerase chain reaction (RT-PCR), confocal indirect immunofluorescent staining, Western blot analysis, and electrophysiological techniques to address these criteria.

Methods

Antibodies and Reagents

Anti-Ro/La antibodies (positive IgG) were obtained from the National Institute of Arthritis and Musculoskeletal and Skin Diseases-supported National Research Registry for Neonatal Lupus (Dr Jill P. Buyon, NYU School of Medicine). In the present study, positive IgG was from 3 mothers whose children have CHB, as tested by ELISA and immunoblot.[4–6,9–11] Negative IgG (control IgG) was from 3 healthy mothers with healthy children who tested negative for anti-Ro/La antibodies.[4–6,9–11] The concentration of positive IgG used was 300 μg/mL for oocyte experiments and 100 μg/mL for tsA201 cells. This was based on data from our previous publications and the dose-response curve in the present study (Figure 5A).[9,10] The anti-α_{1D} antibody is developed in rabbit with a synthetic peptide that corresponds to amino acids 809 to 825 of the α_{1D} subunit of the rat L-type Ca channel (Sigma).

Human Fetal Heart Tissue

Human fetal hearts (15- to 20-week gestation) were obtained after elective termination of normal pregnancy from National Institutes of Health–sponsored tissue banks in Baltimore, Md, and Seattle, Wash. The use of human fetal heart tissue received an exemption from the VA New York Harbor Healthcare System Institutional Review Board.

Rabbit SA/AV Node Isolation

Rabbit SA and AV node tissues were prepared according to Denyer et al[17] and Hancox et al,[18] respectively. All animal protocols were approved by the Institutional Animal Care and Use Committee of VA New York Harbor Healthcare System. Young (6-week-old) New Zealand rabbits were anesthetized with intravenous injection of pentobarbital sodium (50 mg/kg). The heart was rapidly excised and immersed in a normal Tyrode solution containing (in mmol/L): 140 NaCl, 5.4 KCl, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 0.33 NaH_2PO_4, 10 glucose, 5 HEPES (pH 7.4). The SA node, AV node, and atrial and ventricular tissue were snap-frozen in liquid nitrogen.

Reverse Transcription–Polymerase Chain Reaction

Total cellular RNA was isolated from human fetal and rabbit tissue, and reverse transcription was performed as described previously.[19,20] The sense primer was 5′-TTAGTGACGCCTGGAACACG-3′, and the antisense primer was 5′-CCTGTATCAGGAAAGTGG-3′. The primers were chosen from conserved regions among different species and were unique to the α_{1D} Ca channel. The expected PCR amplification size was 1047 bp. Final PCR products were evaluated on ethidium bromide–stained 1% agarose gel. Sequencing of the PCR products was performed by Genemed.

Isolation of Human and Rat Fetal Cardiac Myocytes

Cardiac myocytes were obtained from Langendorff-perfused human fetal hearts as described previously.[4] Hearts were perfused at 37°C with 100% O_2 gassed Tyrode's solution followed by Ca-free Tyrode's solution with 0.5 mg/mL collagenase type B (Boehringer Mannheim). Cells were then dispersed in a Kraft-Brühe solution containing (in mmol/L): potassium glutamate 70, KCl 30, KH_2PO_4 10, $MgCl_2$ 1, taurine 20, glucose 10, HEPES 10. Isolated 17- to 19-day-old fetal Sprague-Dawley rat myocytes were obtained as described previously[21] and cultured on coverslips in Dulbecco's modified Eagle's medium containing 10% calf serum (Gibco) overnight before being subjected to the immunostaining procedures.

Indirect Immunofluorescent Staining

Indirect immunostaining was performed on isolated human and rat fetal myocytes and on $\alpha_{1D}/\beta_{2a}/\alpha_2\delta$-transfected tsA201 cells as described previously.[19,20] Cells were fixed and permeabilized with 4% paraformaldehyde and 0.1% Triton. After they were blocked with 5% normal goat sera, the cells were incubated overnight at 4°C with anti-α_{1D} Ca channel antibody (1:200) and detected with FITC-conjugated anti-rabbit IgG (1:200, Jackson ImmunoResearch Laboratories, Inc). Secondary antibody alone and staining of nontransfected tsA201 cells with anti-α_{1D} antibody were included as negative controls. A confocal scanning laser microscope (MRC-600; Bio-Rad) was used for visualization.

Expression of α_{1D} Ca Channel in tsA201 Cells

tsA201 cells were grown and transiently transfected with 10 μg of a mix of human α_{1D}, rat β_{2a}, and $\alpha_2\delta$ cDNAs (in pCMV6b vector, kindly provided by Drs J. Striessnig, Innsbruck, Austria, and S. Seino, Kobe, Japan) by the calcium phosphate method as described previously.[22] Whole-cell voltage-clamp recording was performed with the Axopatch 200B (Axon Instruments) with pipette resistance of 1.5 to 3 MΩ at 48 hours after transfection. The internal solution contained (in mmol/L): 135 CsCl, 4 $MgCl_2$, 4 ATP, 10 HEPES, 10 EGTA, and 1 EDTA, adjusted to pH 7.2 with TEAOH. The bath solution contained (in mmol/L): 135 choline chlorine, 1 $MgCl_2$, 2 $CaCl_2$, and 10 HEPES, adjusted to pH 7.4 with TEAOH. Signals were sampled at 20 kHz and low-pass filtered at 2 kHz. Data were leak-subtracted online with a P/4 protocol and analyzed with pClamp version 8.0 (Axon Instruments). For α_{1D} I_{Ca-L} current-voltage (I-V) relations, tsA201 cells were depolarized from a holding potential of −100 mV to test potentials between −80 and 60 mV with increments of 10 mV. For the time course, α_{1D} I_{Ca-L} was recorded continuously at a test potential of −10 mV from a holding potential of −100 mV.

Expression and Recording of α_{1D} Ca Current in Xenopus Oocytes

Stage IV and V oocytes were injected with 20 ng of α_{1D}/β_{2a} cRNA encoding the full length of human α_{1D} (subcloned from pCMV6b to pCDNA vector) and rat β_{2a} subunits (in pBS bluescript SK vector,

kindly provided by E. Perez-Reyes, University of Virginia, Charlottesville). Currents were recorded from the fourth to the seventh day after injection.[9,10] The external recording solution contained (in mmol/L): $Ba(OH)_2$ 40, NaOH 50, KOH 2, HEPES 5, 4-AP 5, and TEA 10, pH 7.4. Barium was used instead of calcium to avoid the significant endogenous chloride current in Xenopus oocytes. Oocytes were impaled with electrodes filled with 3 mol/L KCl.

For $I_{Ba\text{-}L}$ I-V relations, oocytes were depolarized from a holding potential of −80 mV to test potentials between −50 and 50 mV with increments of 10 mV. For the time course, $I_{Ba\text{-}L}$ was recorded continuously at a test potential of −10 mV from a holding potential of −80 mV.

Western Blot

tsA201 cells were harvested at 48 hours after transfection with $\alpha_{1D}/\beta_{2a}/\alpha_2\delta$ cDNA. Cells were lysed in a lysis buffer (in mmol/L: Tris/HCl 50, pH 7.4, NaCl 150, EDTA 5, 0.25% Triton X-100, 10% glycerol, NaF 1, Na_3VO_4 1, 10 μg/mL PMSF, aprotinin, leupeptin),[23] and centrifuged at 40 000*g* for 30 minutes. The supernatant (150 μg) was resolved by 4% to 12% SDS/PAGE. Blots were probed with rabbit anti-α_{1D} antibody (Sigma, 1:250) at 4°C overnight and detected with a 1:5000 diluted peroxidase-conjugated anti-rabbit IgG. For the cross-reactivity experiments, after detection, the membrane blot was stripped, split into 2, and then reprobed with positive IgG (1:400) and negative IgG (1:400), respectively. Peroxidase-conjugated anti-human IgG was used for detection. Additional experiments with membrane proteins (150 μg) from human fetal heart and rat fetal heart prepared as described previously[20] were performed to examine expression of α_{1D} Ca channels in these tissues. Membrane protein from adult rat left ventricles was included as negative control.

Statistical Analysis

Statistical comparisons were evaluated with a Student *t* test. Data are presented as mean±SEM. A value of $P<0.05$ was considered significant.

Results

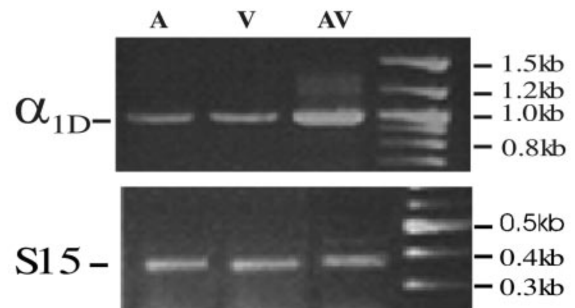
α_{1D} Ca Channel Is Expressed in Human Fetal Cardiac Myocytes

α_{1D} Ca Channel mRNA

CHB is detected in human fetal heart as early as 16 weeks of gestation; however, there are no available data about expression of α_{1D} Ca channel in human fetal heart. We first performed RT-PCR to investigate the expression of α_{1D} mRNA in human fetal hearts (15- to 20-weeks' gestation). Because of the small size and limited access to human fetal hearts, young rabbit SA node tissue was used instead. With an α_{1D} Ca channel–specific primer, α_{1D} Ca channel mRNA was readily amplified from right atria, ventricles, and AV node of human fetal hearts (n=3; Figure 1A). This is in contrast to the young rabbit hearts (n=3), in which α_{1D} Ca channel mRNA was not detected in the ventricles (Figure 1B) but was detected in SA node, atria, and AV node. The brain tissue was used as positive control. A 361-bp band corresponding to the S15 housekeeping gene was seen in all tissues (lower panels, Figures 1A and 1B), which confirmed accuracy in the RNA estimation and gel loading techniques.

α_{1D} Ca Channel Protein

To eliminate possible contamination from noncardiac tissue in the RT-PCR experiments above, indirect confocal immunostaining with anti-α_{1D} Ca channel antibody was performed in isolated human fetal cardiac myocytes (n=3 hearts). Figure 2 illustrates typical immunostaining experiments, with panels A, C, E, and G representing phase controls and panels B, D, F, and H the corresponding staining, respectively. α_{1D} Ca channel protein was localized on the cell membrane of both human right atrial and ventricular myocytes (Figures 2B and 2D). No staining was seen with the secondary antibody alone (Figure 2F). Interestingly, nuclear staining was also observed (Figures 2B and 2D). Because the anti-α_{1D} Ca channel antibody was raised in rabbit against rat α_{1D} Ca channel, we also performed immunostaining experiments using rat fetal ventricular myocytes to eliminate the possible species-related cross-reactivity of the antibody. As in the human fetal heart, both sarcolemmal and nuclear staining were observed in rat fetal myocytes, as shown in Figure 2H. We further tested the staining in both transfected and nontransfected tsA201 cells using the same anti-α_{1D} antibody. In $\alpha_{1D}/\beta_{2a}/\alpha_2\delta$–transfected tsA201 cells, marked staining of α_{1D} Ca channel was observed at the plasma membrane and intracellularly (Figures 3A, 3B, 3C, and 3D). No staining was observed under the same scanning settings with secondary antibody alone (Figures 3E and 3F) or in nontransfected tsA201 cells (Figures 3G and 3H). Human and rat fetal myocytes and transfected tsA201 cells all showed clear sarcolemmal staining. The significance of nuclear staining is not yet known. To complement the immunostaining experiments, Western blots were



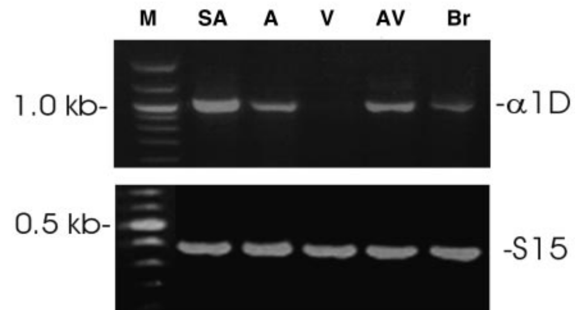


Figure 1. Expression of α_{1D} in human fetal and young rabbit heart by RT-PCR. A, Transcript of 1047 bp corresponding to α_{1D} Ca channel was amplified from human fetal right atrium (A), AV node, and ventricle (V). B, Similar transcript was also amplified from young rabbit SA node, right atrium (A), AV node (AV), and rabbit brain (Br; positive control). No transcript was identified in young rabbit ventricle (V). Bottom panels, 361-bp housekeeping gene S15 was seen in all tissues, which confirms accuracy of cDNA integrity and gel loading techniques. M indicates markers.

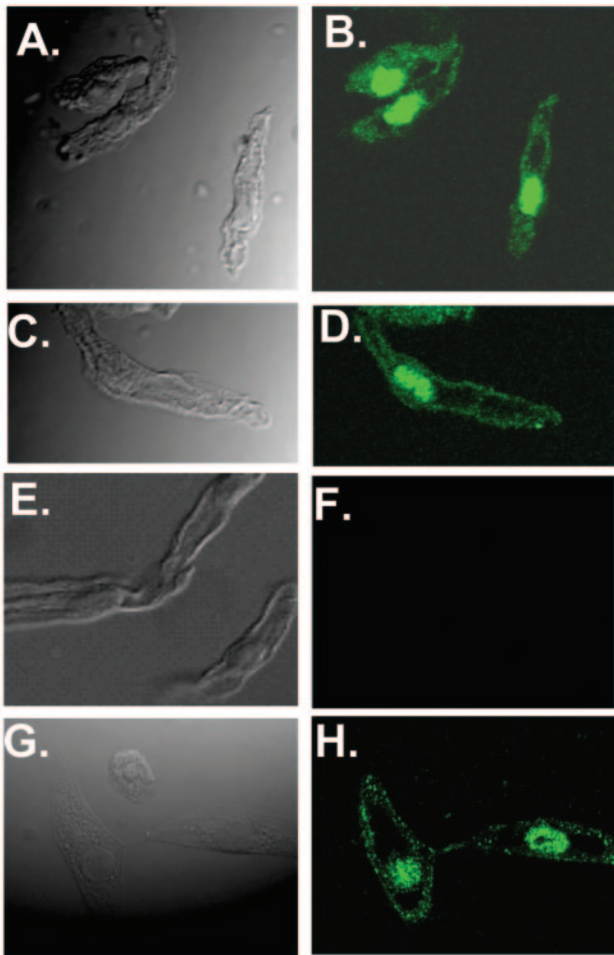


Figure 2. Expression of α_{1D} Ca channel protein in human and rat fetal myocytes. Confocal indirect immunostaining with anti-α_{1D} Ca channel antibody was performed on isolated 15-to 20-weeks' gestation human fetal myocytes and on 19-day-old rat fetal cardiac myocytes. Phase controls showing isolated human fetal atrial myocytes (A), ventricular cells (C and E), and rat fetal ventricular myocytes (G). B, D, and H, Corresponding staining with anti-α_{1D} Ca channel antibody, showing clear membrane and nuclear staining in both atrial and ventricular cells. F, These types of staining were not seen with secondary antibody alone.

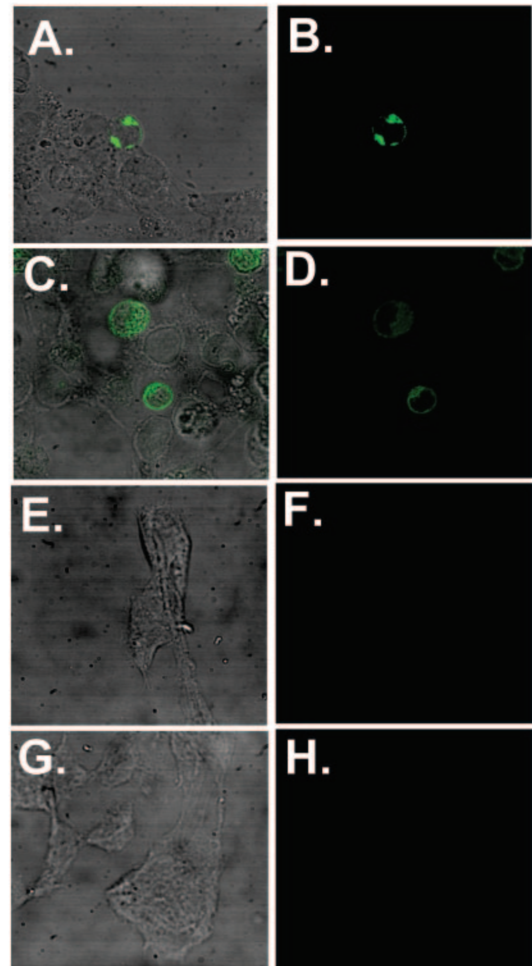


Figure 3. Confocal immunostaining of α_{1D} Ca channel in transfected and nontransfected tsA201 cells. B and D, Staining of $\alpha_{1D}/\beta_{2a}/\alpha_2\delta$-transfected tsA201 cells with anti-α_{1D} Ca channel antibody from 2 different experiments. A and C, Phase controls and overlapping staining. Note specific staining of α_{1D} transfected cells vs absence of staining on neighboring cells that did not express the channel. E and G, Phase controls; F and H, corresponding staining. F, No staining was observed with secondary antibody alone. H, No staining of anti-α_{1D} antibody in nontransfected tsA201 cells under same confocal scanning setting (n=3).

also performed to examine expression of the α_{1D} Ca channel in fetal hearts. Figure 4 shows that the band corresponding to the α_{1D} Ca channel was detected in both human and rat fetal hearts. This band was not seen in the negative control, in which the anti-α_{1D} antibody was preincubated with its antigen peptide for 1 hour at room temperature. Furthermore, no band was observed with proteins from adult rat ventricles, which indicates the specificity of the anti-α_{1D} Ca channel antibody. In summary, the confocal immunostaining and Western blot data establish for the first time that the α_{1D} Ca channel is expressed in the human fetal heart.

α_{1D} $I_{Ca\text{-}L}$ Was Inhibited by Positive IgG and Was Rescued by Bay K8644 in Mammalian tsA201 Cells

Presently, there are no pharmacological agents to separate α_{1D} from α_{1C} $I_{Ca\text{-}L}$ in native cells. Thus, expression systems constitute an alternative for studying the effect of positive IgG on only the α_{1D} Ca channel. Transfection of tsA201 cells with α_{1D} plasmid alone failed to yield any functional channel. This is consistent with a previous report.[24] Similarly, no detectable current was observed in tsA201 cells transfected with $\beta_{2a}/\alpha_2\delta$ subunits alone. Coexpression of α_{1D} together with $\beta_{2a}/\alpha_2\delta$ subunits in tsA201 cells yielded functional α_{1D} $I_{Ca\text{-}L}$ that activated at approximately -60 to -50 mV and peaked at -10 mV with 2 mmol/L Ca used as a charge carrier (Figure 5C). We next tested whether positive IgG would inhibit $I_{Ca\text{-}L}$ and whether this inhibition could be rescued by Bay K8644 (1 μmol/L), a dihydropyridine calcium channel activator. The dose-response curve of positive IgG on α_{1D} $I_{Ca\text{-}L}$ yielded an IC_{50} of 51.4 μg/mL (Figure 5A; n=5). Application of positive IgG (100 μg/mL) reduced the peak of α_{1D} $I_{Ca\text{-}L}$ (Figure 5B). Averaged data showed that inhibition of α_{1D} $I_{Ca\text{-}L}$

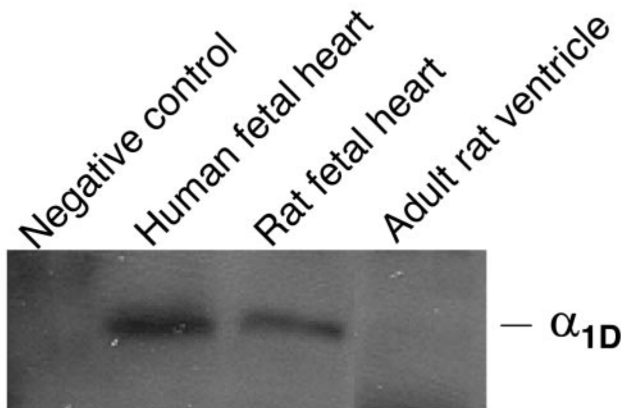


Figure 4. Expression of α_{1D} Ca channel protein in human and rat fetal heart by Western blot. With anti-α_{1D} Ca channel antibody, Western blot data show that band corresponding to α_{1D} Ca channel was detected in both human and rat fetal hearts. This band was not seen in negative control, in which anti-α_{1D} antibody was preincubated with its antigen. No band was observed with proteins from adult rat ventricles.

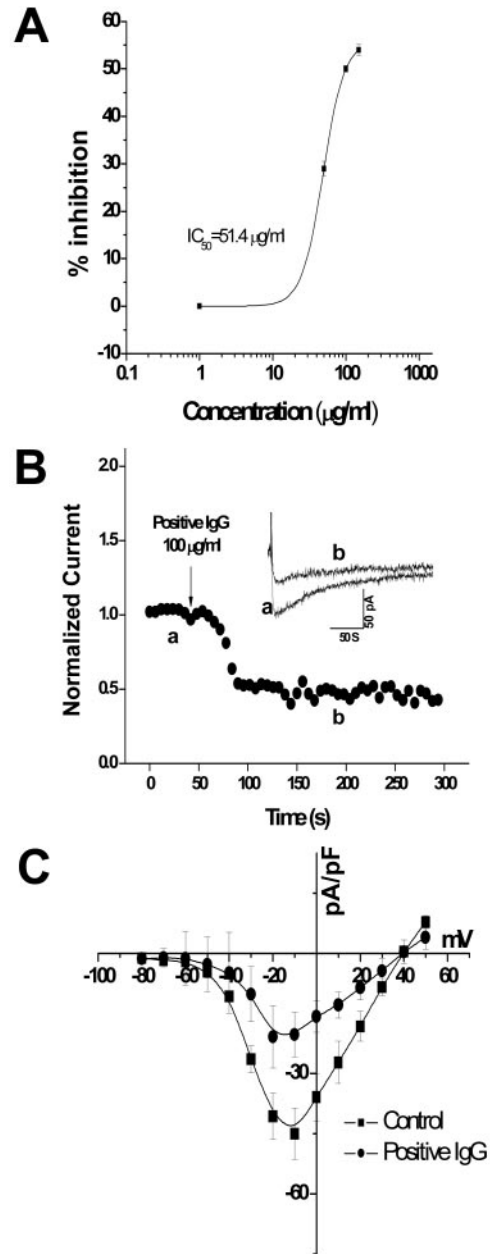


Figure 5. α_{1D} $I_{Ca\text{-}L}$ was inhibited by positive IgG in tsA201 cells. α_{1D} and $\beta_{2a}/\alpha_2\delta$ subunits were cotransfected in tsA201 cells. α_{1D} $I_{Ca\text{-}L}$ was recorded with whole-cell mode of patch-clamp technique with 2 mmol/L Ca as charge carrier. A, Dose-response curve of positive IgG on α_{1D} $I_{Ca\text{-}L}$ yielded IC_{50} of 51.4 μg/mL. B, Time course inhibition of α_{1D} $I_{Ca\text{-}L}$ by positive IgG (100 μg/mL) in 1 tsA201 cell. Current amplitudes were normalized for ease of comparison. Inset shows selected α_{1D} $I_{Ca\text{-}L}$ tracings at time indicated on time course by "a" and "b." C, Averaged I-V curves obtained by depolarizing pulses between −80 and 50 mV from holding potential of −100 mV before and after application of 100 μg/mL positive IgG.

by positive IgG was 42.2±3% at −10 mV (Figure 5C). This inhibition of α_{1D} $I_{Ca\text{-}L}$ was rescued by 1 μmol/L Bay K8644 beyond the baseline level (Figure 6A; note that Bay K8644 was added to positive IgG). The specificity of the inhibition of α_{1D} $I_{Ca\text{-}L}$ by positive IgG was tested by the observation that neither denatured positive IgG (100 μg/mL; Figure 6B) nor the negative IgG (100 μg/mL) had any effect on α_{1D} $I_{Ca\text{-}L}$.

α_{1D} $I_{Ca\text{-}L}$ Was Also Inhibited by Positive IgG and Rescued by Bay K8644 in Xenopus Oocyte

To exclude that α_{1D} $I_{Ca\text{-}L}$ inhibition by positive IgG is not dependent on the expression system, the effect of positive IgG on α_{1D} current was also characterized in Xenopus oocytes. Figure 7A shows I-V relations (panel A) and current traces (inset) of the expressed α_{1D} $I_{Ba\text{-}L}$ in Xenopus oocytes. α_{1D} $I_{Ba\text{-}L}$ activated at approximately −50 to −40 mV and peaked at −10 mV with 40 mmol/L barium as the charge carrier. Figure 7B shows the time course of one representative experiment in which α_{1D} $I_{Ba\text{-}L}$ recorded at −10 mV was inhibited by positive IgG (from 450 nA at control to 325 nA with positive IgG). This inhibition was also rescued by Bay K8644 beyond the baseline level. Averaged data showed that the inhibition of α_{1D} $I_{Ba\text{-}L}$ by positive IgG (300 μg/mL) was 33±10% at −10 mV; Bay K8644 (1 μmol/L) reversed the inhibition of α_{1D} $I_{Ba\text{-}L}$ by positive IgG and further increased the current to 12% above the baseline level (n=6, $P<0.05$; Figure 7C). Negative IgG did not have any effect on α_{1D} $I_{Ba\text{-}L}$.

Positive IgG Cross-Reacts With α_{1D} Ca Channel Protein

We next determined whether α_{1D} $I_{Ca\text{-}L}$ inhibition is due to the direct cross-reactivity of positive IgG with α_{1D} Ca channel protein. Western blot experiments were performed on proteins extracted from $\alpha_{1D}/\beta_{2a}/\alpha_2\delta$-transfected tsA201 cells with positive IgG and anti-α_{1D} antibody as shown in Figure 8. Anti-α_{1D} antibody recognized the 190-kDa band corresponding to the α_{1D} Ca channel protein in all 4 lanes (Figure 8A). The membrane blot was stripped (Figure 8B) and reprobed with positive and negative IgGs, respectively. Positive (Figure 8C) but not negative IgG recognized the same 190-kDa α_{1D} Ca channel protein band (Figure 8D). This confirms that the results are not due to the residual anti-α_{1D} antibody, because no bands were seen on the stripped membrane (Figure 8B). Similar results were found in a total of 3 experiments. Altogether, the data provide convincing evi-

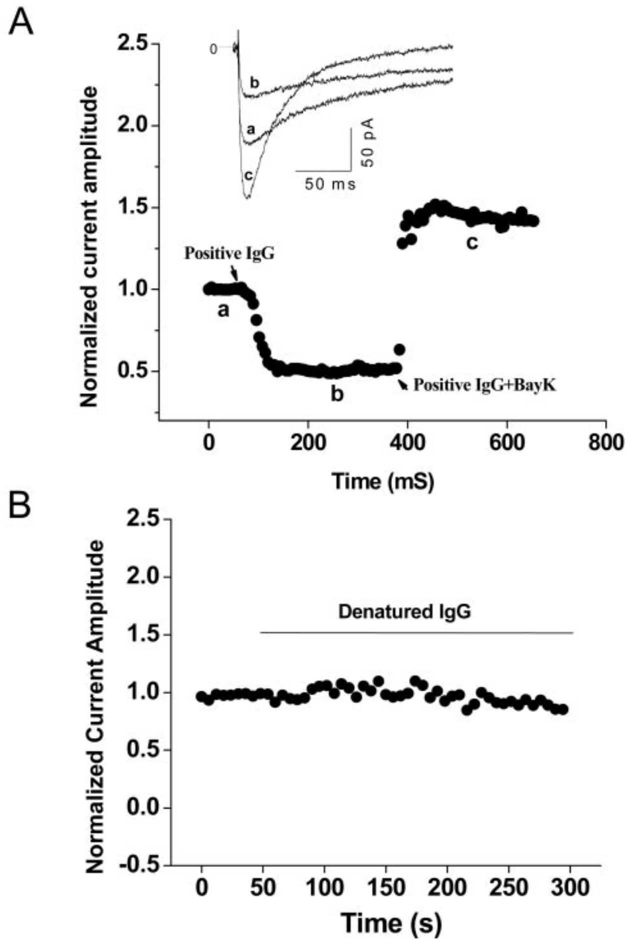


Figure 6. Bay K8644 rescued inhibited α_{1D} I_{Ca-L} by positive IgG. A, Time course of α_{1D} I_{Ca-L} was recorded continuously at test potential of −10 mV from holding potential of −100 mV. Application of positive IgG (100 μg/mL) inhibited α_{1D} I_{Ca-L}, and this inhibition was reversed by Bay K8644 (1 μmol/L). Inset shows selected α_{1D} I_{Ca-L} tracings at time indicated on time course by "a," "b, and "c." B, Time course showing no effect of denatured positive IgG on α_{1D} I_{Ca-L}.

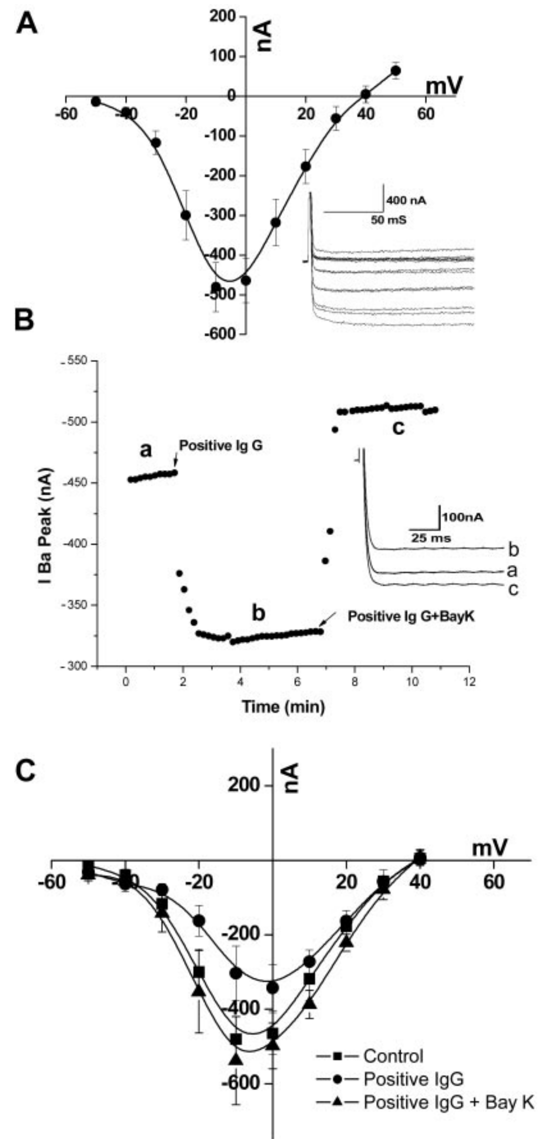


Figure 7. α_{1D} current was also inhibited by positive IgG and rescued by Bay K8644 in Xenopus oocyte. A, Averaged I-V relationships of α_{1D} Ca currents recorded between −50 and 50 mV from holding potential of −80 mV with 40 mmol/L Ba as charge carrier in Xenopus oocytes (n=6). Representative current traces from 1 Xenopus oocyte at voltages between −60 and 50 mV are shown in inset. B, Time course of α_{1D} current during control, after application of positive IgG (300 μg/mL), and in presence of positive IgG plus Bay K8644 (1 μmol/L) in 1 oocyte. Currents were activated by depolarization to −10 mV from holding potential of −80 mV. Selected current traces are shown in inset at times indicated by "a", "b," and "c" on time course of I_{Ba-L}. C, Averaged I-V relationships of α_{1D} current before and after application of positive IgG and positive IgG plus Bay K8644.

dence that positive IgG but not negative IgG directly cross-reacts with Ca channel α_{1D} pore-forming protein.

Discussion

The present data are the first to provide biochemical and functional evidence that the α_{1D} Ca channel is expressed in human fetal heart and that positive IgG from mothers of children with CHB inhibits α_{1D} I_{Ca-L}. Positive IgG from the same mothers used to demonstrate electrophysiological inhibition of α_{1D} I_{Ca-L} also recognized the α_{1D} subunit of the L-type Ca channel by Western blot. Together, the data in this study establish that the α_{1D} Ca channel is a novel target for positive IgG, and consequently, α_{1D} I_{Ca-L} inhibited by positive IgG may account in part for the autoimmune-associated sinus bradycardia seen in CHB.

The causal relationship of positive IgG to autoimmune-associated sinus bradycardia in CHB has been documented in animal models of CHB[4,5,11] and in clinical settings[7,8]; however, the underlying molecular mechanism remains unknown. The candidate antigens SSA-Ro and SSB-La are intracellularly located, and there is no convincing evidence that maternal antibodies can cross the sarcolemma of a normal myocyte. Efforts have therefore been directed toward mechanisms that may cause the translocation of these antigens to the cell surface. Several experimental evidences have shown that viral infection,[25] UV light treatment,[26] and apoptotic death of cells could induce the translocation of Ro/La antigens to the cell surface[27]; however, it is not yet clear what the resulting cellular events and signaling pathways are that

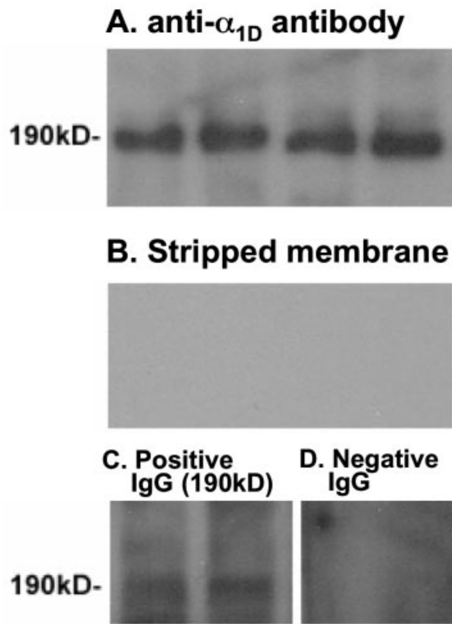


Figure 8. Cross-reactivity of positive IgG with L-type Ca channel α_{1D} protein expressed in tsA201 cells by Western blot. A, α_{1D} Ca channel protein was detected in all 4 lanes with anti-α_{1D} Ca channel antibody (Sigma) on proteins extracted from $\alpha_{1D}/\beta_{2a}/\alpha_2\delta$-transfected tsA201 cells. B, Anti-α_{1D} Ca channel antibody was stripped from membrane. C, Same membrane was split in 2. One part was reprobed with positive IgG. Positive IgG recognized same 190-kDa band corresponding to α_{1D} Ca channel protein. D, Remaining half of stripped membrane was reprobed with negative IgG. Negative IgG did not cross-react with α_{1D} Ca channel protein.

could account for the sinus bradycardia in CHB. Alternatively, another appealing hypothesis, which we termed the "Ca channel hypothesis," proposes that anti-Ro/La autoantibodies cross-react with sarcolemmal components, such as Ca channels, which play an important role in SA node pacemaking activity. This hypothesis is supported by previous observations that Ca channels have been the targets for autoantibodies in other autoimmune diseases.[28–32] It has been reported that autoantibodies against the ADP/ATP carrier of the inner mitochondrial membrane from patients with myocarditis and dilated cardiomyopathy can cross-react with sarcolemmal Ca channel proteins and disturb Ca channel function.[28] Similar cross-reactivity has been reported in other autoimmune disease, such as Lambert-Eaton myasthenic syndrome and lateral sclerosis.[29–32] In addition, we have also previously shown that positive IgG cross-reacts with α_{1C} Ca channel protein and inhibited α_{1C} $I_{Ca\text{-}L}$.[4,6,9–11] This hypothesis is further supported by the present data showing that positive IgG recognized the α_{1D} Ca channel protein and functionally inhibited the expressed α_{1D} $I_{Ca\text{-}L}$.

A number of ion currents have been implicated in SA node pacemaker activity. These include I_f, $I_{Ca\text{-}T}$, $I_{Ca\text{-}L}$, I_K, and I_{Na}. We have previously shown that positive IgG specifically inhibited α_{1C} $I_{Ca\text{-}L}$ and, to a lesser extent, $I_{Ca\text{-}T}$ but did not affect I_f, I_K, or I_{Na}, which indicates specificity for the Ca channel famliy.[4,9–11] However, the inhibition of α_{1C} $I_{Ca\text{-}L}$ by positive IgG cannot account for the reported sinus bradycardia in CHB, because the contribution of α_{1C} $I_{Ca\text{-}L}$ to diastolic depolarization of SA node is generally considered to be minor owing to its more positive activation threshold compared with the spontaneous pacemaker diastolic depolarization.[12] The present data using human fetal heart, together with animal data from other studies, have demonstrated that the previously underappreciated α_{1D} Ca channel appears to play unique and distinct roles in the heart.[14–16] First, unlike the universally expressed α_{1C} Ca channel in the heart, the α_{1D} Ca channel is expressed only in adult SA node, atria, and AV node. Mangoni et al[16] showed $I_{Ca\text{-}L}$ density in SA node cells was decreased by 75% in α_{1D} Ca channel knockout mice compared with wild-type mice, which indicates that the contribution of the α_{1D} Ca channel to total $I_{Ca\text{-}L}$ in the mouse SA node cell is significant. Second, we showed that α_{1D} $I_{Ca\text{-}L}$ was activated at between -60 and -50 mV in tsA201 cells, which falls within the range of pacemaker diastolic depolarization. The fact that the α_{1D} Ca channel is expressed in SA node cells and activates at a low-voltage threshold range and that α_{1D} Ca channel knockout mice exhibit profound sinus bradycardia[14–16] indicates that the α_{1D} Ca channel plays a critical role in maintenance of normal cardiac rhythm. Consequently, inhibition of SA nodal α_{1D} $I_{Ca\text{-}L}$ by positive IgG is expected to slow the heart rate. Indeed, positive IgG-superfused rat and rabbit hearts and pups from mice injected with positive IgG during pregnancy exhibited profound sinus bradycardia by ECG and optical action potential recordings.[4,6,11] Furthermore, we recently showed that superfusion of spontaneously beating single rabbit SA node myocytes with positive IgG resulted in significant sinus bradycardia as measured by an increase in action potential cycle length.[11]

In the present study, we also unexpectedly observed expression of the α_{1D} Ca channel in the fetal ventricle. This fetal ventricular expression of the α_{1D} Ca channel is clinically relevant, because some deaths reported in children with CHB are related to heart failure.[2,33,34] The inhibition by positive IgG of ventricular L-type Ca channels (both α_{1D} and α_{1C}), which are responsible for generating contractile force, will diminish the contraction status of the fetal heart, which depends significantly on sarcolemmal Ca entry. Thus, inhibition of α_{1D} together with α_{1C} $I_{Ca\text{-}L}$ by positive IgG may account for both the sinus bradycardia and contractile impairment seen in CHB infants.

Despite the extensive research effort in CHB, there is no effective pharmacotherapy for CHB to date. If inhibition of $I_{Ca\text{-}L}$ is critical in cardiac impulse generation and conduction found in CHB patients, then an increase in $I_{Ca\text{-}L}$ should rescue or reverse cardiac electrical abnormalities seen in CHB. We demonstrated that application of a Ca channel activator, Bay K8644, reversed the α_{1D} $I_{Ca\text{-}L}$ inhibited by positive IgG beyond the baseline level. This finding is the first experimental investigation in CHB to correlate basic findings to potential therapeutic development of new strategies in the management of CHB. Although Bay K8644 may not be an ideal Ca channel agonist in patients because of its vascular effects, its ability to rescue IgG inhibition of α_{1D} $I_{Ca\text{-}L}$ points to the need to develop new therapeutic agents that will specifically target cardiac Ca channels.

In summary, the data demonstrate that the α_{1D} Ca channel is expressed in human fetal heart, and α_{1D} $I_{Ca\text{-}L}$ is inhibited by

positive IgG by direct binding to the channel protein. Given that the α_{1D} Ca channel plays a critical role in SA node pacemaking and given our previous observation that α_{1C} $I_{Ca\text{-}L}$ and $I_{Ca\text{-}T}$ were also inhibited by positive IgG,[9,10] blockade of α_{1D}, α_{1C} $I_{Ca\text{-}L}$, and $I_{Ca\text{-}T}$ in human fetal heart may contribute to the genesis of sinus bradycardia in CHB.

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