



Autoantibodies from Mothers of Children with Congenital Heart Block Downregulate Cardiac L-type Ca Channels

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Y. QU, G.-Q. XIAO, L. CHEN AND M. BOUTJDIR. Autoantibodies from Mothers of Children with Congenital Heart Block Downregulate Cardiac L-type Ca Channels. *Journal of Molecular and Cellular Cardiology* (2001) 33, 1153–1163. Congenital heart block (CHB) affects offspring of mothers with autoantibodies (positive IgG) to intracellular SSA/Ro and SSB/La ribonucleoproteins and is associated with high morbidity and mortality. Here, we show that maternal anti-Ro/La antibodies immunoreact with human fetal cardiomyocyte sarcolemma, recognize human L-type Ca channel α_{1C} -protein and functionally inhibit expressed current in oocytes injected with α_{1C} cRNA and Purkinje L-type Ca current. Furthermore, cardiac myocytes from pups born to SSA/Ro-immunized mice exhibited reduced L-type Ca current density. All together, the data establish that L-type calcium channel is a target for maternal antibodies and may provide a functional basis for the electrocardiographic abnormalities seen in infants with CHB. © 2001 Academic Press

KEY WORDS: Electrophysiology; IgG; Atrioventricular node; Human fetal myocytes.

Introduction

Congenital heart block (CHB) is a fatal disorder that affects infants born to mothers with autoimmune disease in which maternal autoantibodies are thought to cross the placenta and damage fetal cardiac tissue.¹ It is now well established that CHB detected at or before birth in a structurally normal heart, is strongly associated with autoantibodies (positive IgG) reactive with the intracellular soluble ribonucleoproteins, 52 kDa SSA/Ro, 60 kDa SSA/Ro and 48 kDa SSB/La.^{2,3} CHB is usually detected between 16 and 24-week gestation. Although varying degrees of atrioventricular (AV) block can occur, third degree block (complete AV block) is irreversible and carries a substantial morbidity and mortality approaching 30%, with more than 60% of affected

children requiring lifelong pacemakers.¹ Furthermore, some deaths reported in children with CHB seem to be related to heart failure,^{1,4} suggesting ventricular dysfunction in addition to conduction abnormalities.

We recently established an active animal model for CHB by immunizing female mice with recombinant 52 kDa-SSA/Ro protein^{5,6} and reported conduction abnormalities including complete AV block in the offspring identical to those seen in CHB children. We have also established a passive animal model by directly injecting maternal anti-Ro/La antibodies into pregnant mice and unexpectedly found significant sinus bradycardia in the pups,⁷ indicating that the spectrum of electrical abnormalities extend beyond the AV node to also affect the sinus node. In addition, we reproduced the

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clinical complete AV block in isolated Langendorff-perfused human fetal hearts,⁵ and correlated these findings with L-type Ca channel inhibition in isolated myocytes, by maternal anti-Ro/La antibodies from mothers with CHB children.⁵ None of these abnormalities were seen with antibodies from mothers with healthy children. While these data provide strong evidence supporting an etiologic role of anti-Ro/La autoantibody's involvement in the pathogenesis of CHB, the molecular and ionic basis by which these antibodies cause CHB are not known.

In this study we combined confocal indirect immunofluorescent staining, Western blot analysis and electrophysiological techniques to demonstrate that maternal anti-Ro/La antibodies recognize and directly cross-react with human fetal cardiac L-type Ca channel α_{1C} -protein subunit. We propose that chronic exposure of these channels to antibodies leads to functional downregulation of Ca channels, which play a vital role in the electrogenesis of a normal heart.

Methods

IgG purification and affinity purification of antibodies

Purification of IgG and affinity purification procedures were performed as previously described.^{5,8} In this study, we tested IgG from three mothers whose children have CHB and whose IgG contained anti-52 kDa SSA/Ro, 60 kDa SSA/Ro and 48 kDa SSB/La antibodies, as tested by ELISA and immunoblot.⁸ This is referred to in the text as positive IgG. Negative IgG (control IgG) was purified from sera of three healthy mothers with healthy children, tested negative for anti-SSA/Ro and anti-SSB/La antibodies by ELISA and immunoblot.⁸ The fetal concentrations of total IgG are marginally detectable in the first trimester (<100 mg/dl) and remain low until after 17 weeks, at which time they steadily increase reaching 400 mg/dl by 24 weeks and 800 mg/dl by 32 weeks as placental transfer becomes more efficient.⁹ The level of IgG in CHB cord sera at the time of delivery varied from 500–1500 mg/dl, which corresponds to 5–15 mg/ml. The concentrations that we found to block L-type Ca current in isolated single cardiac myocyte were between 40–100 μ g/ml. Higher concentrations of 800–1200 μ g/ml were required to induce complete AV block in whole heart perfused in a Langendorff fashion.^{5,8} Since the serum specimens were obtained after birth i.e. weeks later after CHB manifestation in the fetus, the exact concentration

of IgG at that time is not known. Therefore the concentration used in this study was based on our previously published work.^{5,8}

Isolation and preparation of cardiac myocytes

Fetal cardiac myocytes were obtained as previously described.^{5,10–12} Briefly human fetal hearts are aseptically obtained after elective termination of normal pregnancy by dilatation and evacuation. This is done in accordance with the guidelines of the Institutional Review Board and after obtaining consent from the mothers. Hearts were transported to our laboratory in Hank's balanced salt solution (HBSS) on ice within 15 min. Cardiac myocytes were obtained from Langendorff-perfused human fetal hearts. Hearts (16–20-week gestation) were perfused at 37°C with a HEPES-buffered solution containing (in mM): NaCl 117, KCl 5.7, NaHCO₃ 4.4, NaH₂PO₄ 1.5, MgCl₂ 1.7, HEPES 20, glucose 11, creatine 10, taurine 20, and 21 U/ml insulin and gassed with 100% O₂, at pH 7.4. After 5 min of wash, the heart was then perfused with fresh buffer mixed with 1mg/ml collagenase type B (Boehringer Mannheim Corp) and 20 μ mol/l CaCl₂ for 5–10 min. The ventricles were then cut off and stirred to obtain cells.

Canine Purkinje cells were obtained as previously described with slight modification.^{13,14} Briefly, dogs were anesthetized with Na pentobarbital (30 mg/kg) and the heart quickly excised from the chest. Purkinje strands from both left and right ventricles were carefully dissected and cut into short segments (2–3 mm) in a cold Ca-free Tyrode's solution. Purkinje strands were agitated in standard HBSS containing 5 mg/ml collagenase (Worthington Type I, Worthington Chemicals, NJ, USA), 5 mM HEPES buffer and 50–70 μ M Ca at 37°C under 100% O₂ shaker bath at a rate of 1–2 cycles/s for about 45–60 min. The digested fibers were incubated for 15 min in (mM) 130 K glutamate, 5.7 MgCl₂, 0.12 EGTA, and 5 HEPES at 37°C. Individual cells were then dispersed by gentle hand pipeting. Cells were stored in normal Tyrode's solution containing 1.8 mM Ca. The research conducted in this study is in conformity with the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals.

Indirect immunofluorescence staining

The freshly isolated fetal cardiac myocytes were fixed in 4% paraformaldehyde in phosphate-

buffered saline (PBS), pH 7.4 for 20 min and permeabilized with 0.1% triton X in PBS for 15 min on ice. Fixed cells were treated with: (1) positive IgG containing, 52 kDa SSA/Ro, 60 kDa SSA/Ro and 48 kDa SSB/La antibodies; (2) affinity-purified 52 kDa SSA/Ro antibody; (3) negative IgG was from mothers of healthy children and tested negative for anti-Ro/La autoantibodies; (4) second antibody alone; (5) antibody against cardiac myosin heavy chain (MHC). Cells were incubated with the above antibodies (1:50) in PBS for 1 h, followed by incubation in blocking buffer containing 5% goat serum and 0.1% BSA in PBS for 60 min and detected with FITC- or TRITC-conjugated goat anti-human IgG. Scanning laser confocal microscope system was used to determine the cellular localization of the labelled antigens.

Partial purification and immunoprecipitation of L-type Ca channel α_{1C} -subunit

Partial purification of Ca channel was carried out according to Rengasamy *et al.*¹⁵ with some modification. Briefly, 10 g of ventricles from human fetal hearts (16–20 week gestation) were homogenized in 0.25 M sucrose with 10 mM histidine (pH 7.4). This and all subsequent buffers included the following cocktail of protease inhibitors: 0.2 mM PMSE, 0.1 mM benzamide, 1 μ M pepstatin A, 0.1 μ M aprotinin, 1 μ M leupeptin, 8 μ g/ml calpain inhibitors I and II, 2 mM EDTA, 0.1 mM EGTA. Large pieces of tissue were removed by low-speed centrifugation. The supernatant was loaded onto a three-layer sucrose cushion of 15, 32 and 40%, respectively, and spun at 28 K for 3 h in a Beckman Ultracentrifuge. A faint band at the 32%/40% sucrose interface was recovered and centrifuged at 24 K for 30 min, the resulting pellet was resuspended in 10 mM Tris, 0.1 M NaCl, 1% digitonin. This sample was shaken on ice for 1 h, spun at 26 K for 30 min, following which the supernatant was recovered. Six μ l/ml of anti- α_{1C} -subunit of Ca channel antibody was added to the supernatant, which was precleared with protein A-sepharose at 4°C overnight. Thirty μ l of 50% sepharose suspension beads were added for every 1 ml of sample and incubated at 4°C for 4 h. The protein A-sepharose antibody/antigen complex was collected by centrifugation, washed, and eluted in non-reducing SDS sample buffer by boiling for 5 min.

Western blot

Western blot of the above samples was performed as previously described:¹⁰ 15 μ l/lane of above eluted

antigens were subjected to SDS-PAGE under non-reducing conditions. Proteins were transferred to PVDF membrane, blocked for 2 h in 5% non-fat milk and 0.3% Tween-20™, incubated with positive IgG (1:400) and anti-Ca²⁺ channel α_{1C} -subunit antibody (1:1000, Alamone Labs, Israel) for overnight at 4°C respectively. Control IgG that tested negative for anti-Ro/La autoantibodies was also studied. Immunodetection of the primary antibodies were carried out with a 1:5000 diluted peroxidase-conjugated anti-human and anti-rabbit IgG for 60 min respectively, and detected with enhanced chemiluminescence (ECL) kit.

Preparation of *Xenopus* oocytes and cRNA injection

Mature female *Xenopus* frogs, purchased from Xenopus I (Ann Arbor, MI, USA), were anesthetized with 1.5 mg/ml tricaine. Surgically removed ovarian lobes were dissected and treated for 1.5 h with 1.5 mg/ml collagenase type IA dissolved in Ca-free ND96 medium (mM: NaCl 96, KCl 2, MgCl₂ 2, HEPES 5, pH = 7.4). Stage IV and V oocytes were selected. cRNAs encoding the full length of α_{1C} -subunit of rabbit cardiac Ca channel was kindly provided by Dr Mohamed Chahine from Laval Hospital Research Center, Quebec, Canada.¹⁶ Each oocyte was injected with 55 nl volume containing 20 ng α_{1C} cRNA. The injected oocytes were stored at 18°C in Leibovitz's L-15 medium (GIBCO BRL, MD, USA) supplemented with 50 U/ml penicillin/streptomycin. Currents were recorded from the 4th to the 7th day after injection.

Oocyte current recordings

The composition of external recording solution for L-type current is (mM): Ba(OH)₂ 40, NaOH 50, KOH 2, HEPES 5, 4-aminopyridine (4-AP) 5, and TEA 10, adjusted to pH = 7.4 with methansulfonic acid. The expressed macroscopic currents were recorded with two-electrode voltage clamp technique using GeneCLAMP 500 amplifier (Axon Instrument Inc., Foster City, CA, USA). The volume of the recording chamber was 0.5 ml, and the rate of perfusion was 0.3 ml/min. Oocytes were impaled with electrodes filled with 3 M KCl in ND96 external solution. Oocytes with membrane potential equal or more negative than -40 mV were used for current recording. For L-type Ba (I_{Ba}) current-voltage (I-V) relations, oocytes were depolarized from a holding potential of -80 mV to test potentials ranging from

–50 to 60 mV, with increments of 10 mV. Time course of L-type I_{Ba} was recorded by a depolarization pulse to 10 mV from a holding potential of –80 mV.

Immunization

Female mice were immunized as previously described.^{5,6} Briefly, mice were injected intraperitoneally and intracervically with 100 μ g of recombinant 52 kDa SSA/Ro protein in Freund's adjuvant at 10-day intervals. The age-matched vehicle group was injected with only Freund's adjuvant solution at similar intervals. Offspring were obtained by breeding female mice with syngeneic males.

Solutions for whole cell patch clamp experiments in mouse ventricular myocytes

Single cardiac myocytes were obtained from mice whose mothers were immunized with 52 kDa SSA/Ro protein, using the same enzymatic dissociation procedure as that for human fetal heart (see above). The composition of external solutions to record whole cell L-type Ca current, I_{CaL} ^{5,8} is (in mM): NaCl 132, CsCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.8, NaH_2PO_4 0.6, 4-AP 5, HEPES 10, dextrose 5, Na-pyruvate 5, pH 7.4. Patch electrodes were filled with internal solution containing (in mM): CsCl 139.8, K-EGTA 10, $MgCl_2$ 4, $CaCl_2$ 0.062, Na_2 -creatine phosphate 5, HEPES 10, Na_2 ATP 3.1, Na_2 GTP 0.42, adjusted to pH 7.1 with KOH.

Current recordings from mouse ventricular and canine Purkinje myocytes

Whole cell voltage clamp protocols were previously reported.^{5,8} Briefly, to record L-type I_{CaL} , all K currents were blocked with intracellular and extracellular Cs and 4-AP^{5,8} and T-type Ca current was blocked by $NiCl_2$. The fast Na current was blocked by a prepulse to –50 mV from a holding potential of –80 mV in the presence of tetrodotoxin (50 μ M) to eliminate any residual Na current. Cells were depolarized every 10 s from a holding potential of –80 mV to a prepulse level of –40 mV for 100 ms

and subsequently to a test pulse of 0 mV for 300 ms. All experiments were performed at room temperature (22–24°C).

Data analysis

Data are presented as mean \pm S.E.M. *t*-test was used when appropriate. A *P* value of <0.05 was considered statistically significant.

Results

Biochemical studies

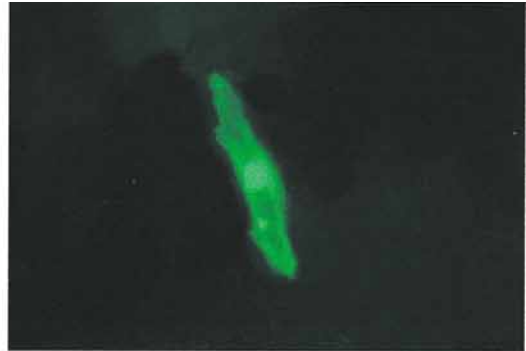
Positive IgG immunostained the sarcolemma of human fetal cardiac myocytes

To determine whether positive IgG from mothers with CHB children cross-react with antigenic determinants at the cell sarcolemma other than the intracellular cognate antigens, we used confocal imaging with FITC or TRITC labeled secondary antibody on isolated human fetal cardiomyocytes (16–20 weeks gestation). Panels A, C, E, and G represent phase contrast and panels B, D, F and H represent the corresponding immunostaining in Figure 1. Cells labeled with positive IgG containing anti-SSA/Ro and anti-SSB/La antibodies showed sarcolemmal and bright nuclear staining (panel B). Cells treated with affinity purified anti-52 SSA/Ro antibodies showed patchy sarcolemmal and some nuclear staining (panel D). These types of staining were not seen with negative IgG (panel F). Similar results were obtained with antibodies from all the three mothers and in a total of 210 myocytes from six hearts. Panel H shows that cells used in this study are cardiac myocytes with clear striations [this is obvious even in myocytes without the staining (phase)]. Secondary antibody alone did not show any specific staining (data not shown). These data suggest that maternal anti-Ro/La antibodies from mothers with CHB children recognize antigenic targets at the human fetal myocyte sarcolemma in addition to the expected intracellular Ro/La antigens. We next tested the hypothesis that Ca channel subunits and/or associated regulatory

Figure 1 Confocal imaging with FITC- or TRITC-labeled second antibody on isolated human fetal cardiomyocytes. Myocytes from human fetal hearts (16–20-week gestation) were treated with primary antibodies as follows: (1) positive IgG containing anti-SSA/Ro and anti-SSB/La antibodies (Abs); (2) affinity purified anti-52 SSA/Ro antibody (Ab); (3) negative IgG (containing no anti-SSA/Ro and anti-SSB/La antibodies); (4) anti-myosin heavy chain antibody. Panels A, C, E, and G represent phase contrast and panels B, D, F and H represent the corresponding immunostaining.



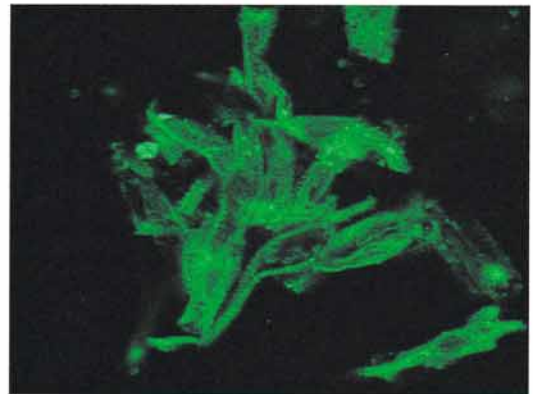
A. Phase



B. Positive IgG (Anti-Ro/La Abs)



C. Phase



D. Affinity purified anti- Ro52 Ab



E. Phase



F. Negative IgG



G. Phase



H. Anti-MHC Ab

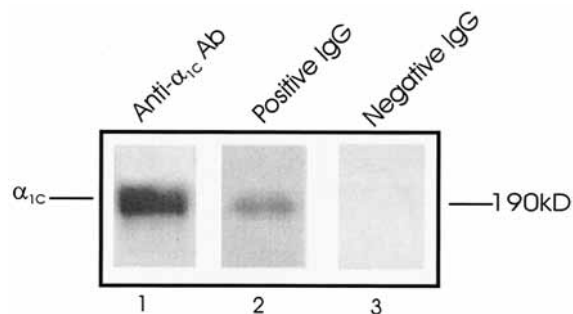


Figure 2 Immunoblot of human fetal cardiac Ca channel α_{1C} -subunit. Immunoblots were performed under non-reducing conditions on 8–16% Tris-Glycine gel. Human fetal hearts (16–20-week gestation) were used. Immunoprecipitated proteins with anti- α_{1C} antibody were separated by SDS-PAGE, transferred to PVDF membrane and then probed with anti- α_{1C} antibody (lane 1), positive IgG (lane 2) and negative IgG (lane 3).

proteins could be a possible target for maternal antibodies.

Positive IgG cross-reacted with proteins immunoprecipitated by anti-L-type Ca channel α_{1C} -subunit antibody

To demonstrate unambiguously that positive IgG cross-reacts with plasma membrane Ca channels, we partially purified Ca channel α_{1C} -subunit from human fetal myocardium by gradient sucrose centrifugation and subsequent isolation by immunoprecipitation with specific anti- α_{1C} antibody. Immunoprecipitated proteins were separated with SDS-PAGE under non-reducing condition and probed with positive IgG from two mothers and anti- α_{1C} antibody, respectively, to examine the possible cross-reactivity (Fig. 2). The anti- α_{1C} antibody, which recognizes both the high molecular weight (210 kDa) and the low molecular weight (190 kDa) of the Ca channel α_{1C} -subunit, recognized essentially the smaller 190 kDa protein (Lane 1). When the same protein was probed with positive IgG, a band (Lane 2) at the same 190 kDa size as that of anti- α_{1C} antibody is seen indicating cross-reactivity with purified Ca channel α_{1C} -subunit. Negative IgG lacking anti-SSA/Ro and anti-SSB/La antibodies did not recognize the 190 kDa protein (Lane 3). Similar results were found in five other experiments. The data provide the most compelling evidence that positive IgG, but not negative IgG directly cross-react with Ca channel α_{1C} pore-forming protein.

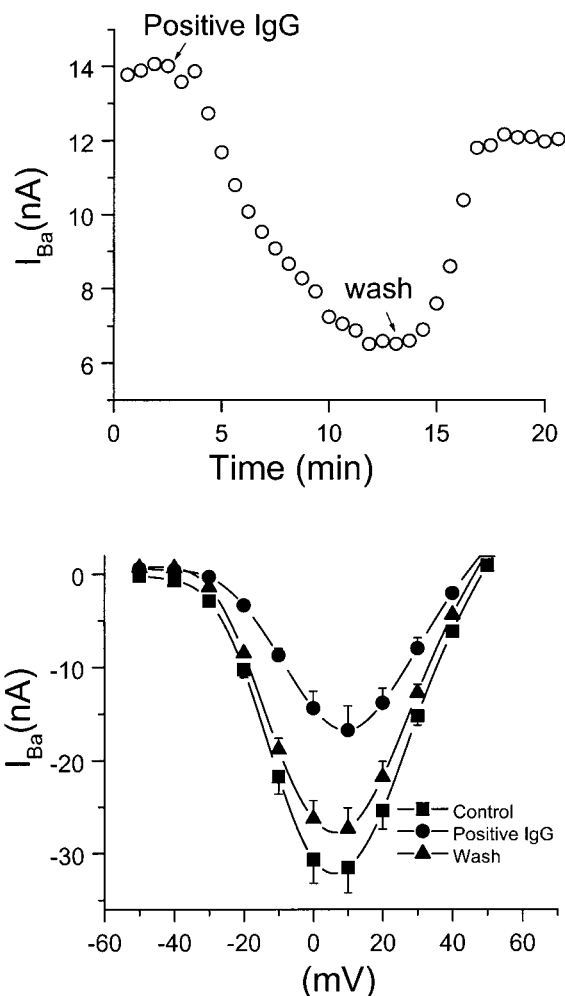


Figure 3 Effects of positive IgG on expressed L-type I_{Ba} recorded from *Xenopus* oocytes. Upper panel shows the time course of the inhibitory effect of positive IgG (350 μ g/ml) on L-type I_{Ba} from one oocyte. Lower panel shows the steady state effect of positive IgG on current–voltage relations of I_{Ba} in seven other oocytes.

Electrophysiological studies

L-type I_{Ba} was inhibited by positive IgG

To determine the functional consequences of cross-reactivity of positive IgG with Ca channel proteins, we next expressed the pore-forming α_{1C} -subunit of L-type Ca channel encoding rabbit cardiac dihydropyridine receptor in *Xenopus* oocytes. Whole cell Ba (40 mM) current, I_{Ba} was recorded in Cl- and Ca-free bath solutions using standard double-electrode voltage clamp technique. Figure 3 (upper panel) shows the time course inhibition of I_{Ba} by positive IgG in one typical oocyte. Application of positive IgG (350 μ g/ml) resulted in 52% inhibition of I_{Ba} ($P < 0.01$) at 10 mV. The effects of positive IgG were only partially reversible. Figure 3 (lower panel)

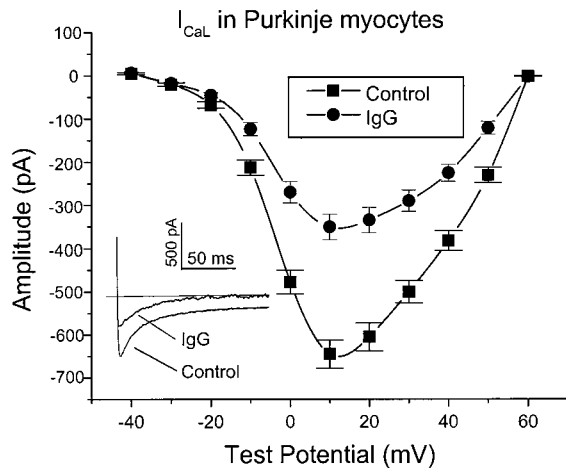


Figure 4 Effects of positive IgG on L-type I_{Ca} recorded from Purkinje cardiac myocytes. Current–voltage relations of I_{CaL} recorded from canine Purkinje cardiac myocytes during control and after the application of positive IgG (100 μ g/ml). Selected current tracings at 10 mV during control and after positive IgG are shown in the inset.

illustrates the current–voltage relations for I_{Ba} before and after the addition of positive IgG and wash out. Positive IgG (350 μ g/ml) inhibited I_{Ba} by $49.6 \pm 4.5\%$ (at 10 mV, $P < 0.01$, $n = 7$). However, negative IgG had no significant effect on I_{Ba} ($n = 6$, data not shown). These data are consistent with those obtained with native L-type Ca current in myocytes^{5,8} and indicate a direct functional interaction of positive IgG with the pore-forming α_{1C} -subunit.

L-type I_{Ca} from Purkinje fibers was inhibited by positive IgG

To assess whether maternal antibodies affect L-type Ca channels from the conduction system, such as the Purkinje fibers, we recorded I_{CaL} from canine Purkinje cardiac myocytes and tested the effects of maternal IgG. The currents were recorded under conditions that suppressed Na, K and T-type Ca currents (see Methods). Data were obtained from a holding potential of -40 mV and depolarizing potentials ranging from -40 to 60 mV in increments of 10 mV. Positive, but not negative IgG (100 μ g/ml) significantly inhibited I_{CaL} by $50.5 \pm 6.5\%$ ($P < 0.01$, $n = 6$). Figure 4 shows an I–V relation of L-type Ca current recorded from Purkinje cells before and after the addition of positive IgG (100 μ g/ml).

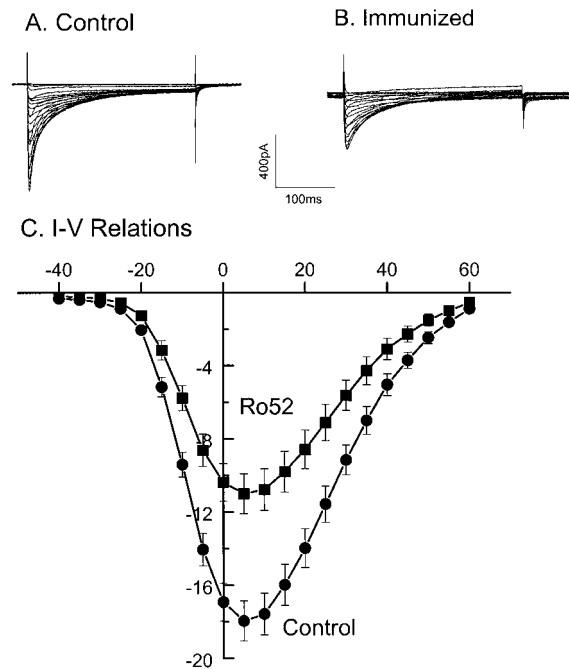


Figure 5 L-type Ca channel current and its density in pup ventricular cells from control and immunized (Ro52) mice. Panel A and B show I_{CaL} elicited by a series of depolarizing 300-ms pulses from -40 mV to 60 mV, with 5 mV increments. Recordings A and B are from the control and Ro52 group respectively. Panel C illustrates mean density current–voltage relations for control ($n = 17$) and Ro52 group ($n = 24$). Peak inward current amplitude was normalized to cell capacitance and plotted against test potential. Values in panel C are mean \pm S.E.M.

Reduced L-type Ca current density in mouse heart with autoimmune associated conduction abnormalities

While the above biochemical and electrophysiological findings clearly indicate that maternal anti-Ro/La antibodies interact directly with L-type Ca channels to inhibit their function, the mechanism by which these antibodies cause cardiac dysfunction is not known. To address this issue, we used our previously established murine model for CHB where mice immunized with recombinant 52 kDa-SSA/Ro protein gave birth to pups with varying degrees of conduction abnormalities,⁵ to test the hypothesis that prolonged exposure of pups heart to maternal antibodies during pregnancy could lead to reduced Ca channels density. We thus examined macroscopic Ca currents recorded in pups from immunized and control mothers. As shown in Figure 5(c), I_{CaL} density in 52 kDa-Ro-immunized group was reduced by $38.6 \pm 4.5\%$ ($P < 0.02$, $n = 17$ for control and $n = 24$ for 52 kDa-Ro). The reduced I_{CaL} density could be attributed to a decrease in the number of functional Ca channels probably

due to internalization and degradation during prolonged exposure *in utero* to maternal antibodies.

Discussion

The present data are the first to provide biochemical and functional evidence that anti-SA/Ro and SSB/La antibodies from mothers of children with CHB directly cross-react with human fetal heart L-type Ca channel α_{1C} -subunit. Antibodies from the same mothers used to demonstrate electrophysiological inhibition of L-type Ca channels also recognized α_{1C} -subunit of L-type Ca channels by Western blot. These results are consistent with the finding that L-type Ca channel density is reduced in hearts of pups chronically exposed *in utero* to maternal antibodies. This could account for some of the electrocardiographic abnormalities seen in infants born to mothers with anti-SSA/Ro and anti-SSB/La antibodies since L-type Ca channels play a major role in slow action potential propagation and excitation-contraction coupling.

Intracellular autoantigens to maternal antibodies

CHB is strongly associated with maternal anti-SSA/La and SSB/Ro antibodies. The intracellular SSA/Ro and SSB/La ribonucleoproteins and their cognate antibodies have been extensively characterized at the molecular level. Sixty kDa SSA/Ro contains a putative zinc finger and an RNA-binding protein consensus motif.¹⁷ Many sera which recognize 60 kDa SSA/Ro protein also react with another protein of 52 kDa (SSA/Ro), comprising three distinct domains: an N-terminal region with three zinc fingers, a central region with a leucine zipper motif, and a C-terminal "rfp-like" domain.¹⁸ Anti-SSB/La antibodies recognize a 48 kDa polypeptide that does not share antigenic determinants with either 52 kDa or 60 kDa SSA/Ro.¹⁹ The exact role of these intracellular antigens and their antibodies in the pathogenesis of CHB is not yet well understood.

Other potential antigenic targets to maternal antibodies

The inaccessibility of maternal antibodies to their intracellular antigens, in normal cells, has raised the possibility that maternal autoantibodies may cross-react with some antigenic targets other than intracellular Ro/La autoantigen. Antibodies from mothers, whose children had CHB were reported to cross-react with laminin B1 and to human cardiac

myosin heavy chain.²⁰ Computer-based analysis of the amino acid sequence from the SWISS-PROT database between 52 kDa SSA/Ro, 60 kDa SSA/Ro and 48 kDa SSB/La proteins and the α_{1C} -subunit of L-type human cardiac sarcolemmal Ca channels revealed that most of the homology is with 52 kDa SSA/Ro, with minor homology to 60 kDa SSA/Ro and 48 kDa SSB/La. When the amino acid sequence is compared in a linear representation, the maximum number of homologous amino acids between the 52 kDa SSA/Ro and α_{1C} -subunit of L-type Ca channel do not exceed four amino acids. However, when a 3-dimensional representation of the α_{1C} -subunit structure was used, numerous homologous epitopes are found in the extracellular loops. Epitopic regions on the α_{1C} -subunit could then be identified; one of which corresponds primarily with the pore-forming region between segments S5 and S6 of domain VI. This supports the present findings of direct interaction between maternal antibodies and sarcolemmal L-type cardiac Ca channel α_1 -subunit. The exact site(s) of interaction remain to be determined. Recently, Eftikhari *et al.*²¹ affinity-purified anti-G12V autoantibodies from lupus patients and showed that they recognize both the 5-HT₄ receptor and the rSSA/Ro protein in a specific manner indicating the existence of cross-reactive epitopes. Using the patch clamp technique, they further showed the ability of anti-G12V antibody to antagonize serotonin-induced I_{CaL} activation in adult human atrial myocytes. They proposed that blockade of 5-HT₄ receptors could lead to reduction of calcium channel activation and even possibly the pacemaker "I_T" current channel leading to conduction abnormalities. Therefore, maternal antibodies could either directly (by interacting with Ca channel protein) or indirectly (through receptors that modulate channels) alter ion channel function.

Proposed pathophysiological mechanisms of CHB

As the candidate antigens are intracellular and there is no convincing evidence that maternal antibodies can cross the sarcolemma of a normal cell, effort has been directed towards mechanisms that may cause the translocation of these antigens to cell surface membrane. The conventional wisdom is that these antigens must be accessible to maternal antibodies to explain the pathological events, mainly the inflammatory process that could lead to CHB. Several experimental evidences have been proposed to account for the accessibility of antigens to maternal antibodies. These include: (1) viral

infection of cells to induce the antigens translocation to the cell surface;²² (2) the expression of SSA/Ro and SSB/La antigens on the keratinocyte cell surface by ultraviolet light;²³ (3) 17 β -estradiol-enhanced binding of anti-SSA/Ro and anti-SSB/La antibodies to keratinocytes;²⁴ (4) autopsy studies showing that maternal IgG was associated with specificity for the SSB/La antigen on the surface of fetal myocardial fibers;²⁵ (5) unknown inductive events *in utero* which may lead to apoptotic cell death, thus exposing the antigens.²⁶ While autoantigens translocation to cell surface could be demonstrated, it is not yet established what are the resulting cellular events and signaling pathways that could account for the electrophysiological abnormalities associated with CHB.

The focus on delineating the basic mechanisms of CHB from an electrophysiological viewpoint is just emerging. First, Alexander *et al.*²⁷ demonstrated that IgG fraction of anti-SSA/Ro SSB/La-positive maternal sera shortened neonatal rabbit cardiac action potential repolarization. Then, Garcia *et al.*²⁸ showed that electrocardiographic conduction disorders associated with neonatal lupus could be reproduced in an isolated adult rabbit heart. Further, they showed that anti-SSA/Ro positive sera inhibited I_{CaL} in isolated ventricular myocytes. Subsequently we established both an active^{5,6} and a passive⁷ animal model of the human CHB, showed that maternal autoantibodies can induce complete AV block in Langendorff-perfused beating heart and in isolated AV multicellular preparations.^{5,8} In addition, we correlated these findings with the inhibition of I_{CaL} by the antibodies in isolated cardiac myocytes from both the working myocardium^{5,8} and the conduction system (Fig. 4).

An apparently unresolved paradox concerns the mechanism by which blockade of L-type Ca channel *in utero* translates into CHB. Fetal heart Ca channels are chronically exposed to maternal antibodies during pregnancy (starting at about the 12th week of gestation when significant amounts of IgGs are detected in fetal circulation). Here, we propose that this chronic exposure to maternal IgG could lead to binding, subsequent internalization, degradation of L-type Ca channels and eventually cell death since L-type Ca channels are known to play a vital role in fetal excitation-contraction coupling.²⁹ This hypothesis is further supported by the present data demonstrating that reduced density of functional L-type Ca channels in pups of Ro 52 immunized mothers. Additional evidence in support of the present findings, is the observation that some deaths reported in children with CHB are related to heart failure.^{1,4} This is likely because of maternal anti-

bodies' inhibition of ventricular L-type Ca channels, which are responsible for generating the contractile force, will exacerbate and worsen the contractile status of the heart. Thus, the Ca channel blockade hypothesis would account for both the chronic and acute effects of maternal antibodies. Blockade of AV nodal myocyte L-type Ca channels could explain the *in vivo* AV block and ventricular myocyte L-type Ca channel blockade could explain, at least in part, the heart failure reported in infants with CHB. However, we recognize that the "Ca channel blockade hypothesis", as any other hypothesis, does not explain all aspects of CHB, including the discordance in twins and the low prevalence of the disease, suggesting that other factors and avenues should be explored.

Vulnerability of fetal heart v mother's heart

Although antibodies to components of the SSA/Ro-SSB/La ribonucleoproteins complex are essential to the development of CHB in offspring, it is intriguing that there are almost no reports of third-degree AV block in mothers despite exposure to identical circulating antibodies. Interestingly, two independent studies^{30,31} reported simultaneous existence of first-degree AV block in the same anti-SSA/Ro positive mother and CHB in her child. This emphasizes the complex pathogenesis of CHB because even if the mother develops heart block, the degree is invariably less severe. Several explanations have been proposed. These include: (1) the presence of a fetal factor e.g. there is a unique antigenic exposure related to a specific time period of cardiac embryogenesis and contact with placentally transferred maternal antibody;³² (2) the possibility that complement regulatory molecules may be diminished on the surface of fetal cells is another explanation; (3) protective maternal hormonal factors were also evoked,³³ (for example estradiol has been reported to enhance binding of anti-SSA/Ro and anti-SSB/La antibodies to keratinocytes but not to cardiocytes);^{23,34} and (4) Eftekhari *et al.*^{21,35} reported that 5-HT₄ receptors could be more important in the developing heart than the adult heart. The assumption is that 5-HT₄ receptors could functionally substitute β -adrenergic receptors during development. Alternatively, our explanation is based on the observation that I_{CaL} density is higher in the mother than the fetal heart. This is supported by several lines of evidence: (a) adult heart I_{CaL} current density^{36,37} is two- to four-fold higher than the human fetal heart;^{5,38} (b) adult heart L-type Ca

channel α_{1C} subunit mRNA levels are four- to nine-fold higher than the human fetal heart;³⁹ furthermore, (c) the clinical observation that fetal cardiac muscle is more sensitive to Ca channel blockers is likely due to the low levels of Ca channel expression in the fetal heart.⁴⁰ It has been suggested that during fetal and neonatal stages, when expression of Ca channels involved in EC coupling is low, small doses of channel blockers could have adverse effects on the immature myocardium.⁴⁰

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