Direct Inhibition of Expressed Cardiac L- and T-Type Calcium Channels by IgG From Mothers Whose Children Have Congenital Heart Block

Guang-Qian Xiao, MD; Keli Hu, MD, PhD; Mohamed Boutjdir, PhD

Background—Congenital heart block (CHB) is a disease that affects the offspring of mothers with autoimmune diseases.

We recently reported that maternal sera containing antibodies against SSA/Ro and SSB/La ribonucleoproteins (positive IgG) inhibited L-type Ca current in isolated cardiac myocytes and induced sinus bradycardia in a murine model of CHB. The direct interaction of positive IgG with L-type Ca channel proteins and the possible inhibition of T-type Ca current that could account for the sinus bradycardia remain unknown.

Methods and Results—The 2-electrode voltage-clamp technique was used to record currents via L-type (I_{Na}\alpha_{1C} or I_{Na}\alpha_{1C}\beta_25+\alpha_3/\delta) and T-type (I_{Na}\alpha_{1T}) Ca channels, Na channels (I_{Na}-hH1), and K channels (I_{Kc}-minK+KvLQT1) expressed in Xenopus oocytes. Positive IgG (350 μg/mL) inhibited I_{Na}\alpha_{1C} by 50.6±4.7% (P<0.01) and I_{Na}\alpha_{1T} by 50.9±4.2% (P<0.01); I_{Na}\alpha_{1H} was reduced by 18.9±1.0% (P<0.01). Immunoblot data show cross-reactivity of positive IgG with α_{1C} subunit. Pretreatment of oocytes with atropine (1 μmol/L) or acetylcholine (10 μmol/L) did not affect the inhibitory effect of IgG on I_{Na}\alpha_{1C} and I_{Na}\alpha_{1T}+\beta_25+\alpha_3/\delta (P<0.05). Positive IgG had no effect, however, on either I_{Na}-hH1 or I_{Kc}-minK+KvLQT1.

Conclusions—Positive IgG inhibited expressed L-type I_{Na} and cross-reacted with the α_{1C} subunit in Xenopus oocytes, providing strong evidence that maternal antibodies interact directly with the pore-forming α_{1C} subunit of Ca channels. In addition, we show for the first time that positive IgG also inhibited T-type I_{Na} but not I_{Na}-hH1 or I_{Kc}-minK+KvLQT1. This could provide, in part, the ionic basis of sinus bradycardia reported in animal models of CHB and clinically in humans. (Circulation. 2001;103:1599-1604.)

Key Words: antibodies ■ ion channels ■ electrophysiology

Congenital heart block (CHB) is a disease that affects children of mothers who may have autoimmune disease or may be entirely asymptomatic. CHB is usually detected between 16 and 24 weeks of gestation in fetuses with otherwise normally developing hearts. CHB carries high mortality (≈30%), and >60% of affected children require pacemakers. Although varying degrees of block have been noted and second-degree block has on rare occasions reverted to normal sinus rhythm, CHB is irreversible. CHB detected in utero is strongly associated with autoantibodies reactive to the intracellular ribonucleoproteins SSA/Ro and SSB/La. Anti-SSA/Ro antibodies recognize 2 proteins: a 60-kDa protein and a 52-kDa protein. An additional 75-kDa phosphoprotein was recently reported to be associated with 60-kDa SSA/Ro. The 60-kDa SSA/Ro protein contains an RNA-binding protein consensus motif. The 52-kDa SSA/Ro protein has 3 distinct domains: 2 zinc fingers in the N-terminal, a central leucine zipper, and a C-terminal rfp-like domain. SSB/La is a 48-kDa protein, which is thought to have the function of facilitating the maturation of RNA polymerase III transcripts. The exact function of these autoantigens is yet to be defined.

The association between CHB and autoantibodies against SSA/Ro and SSB/La proteins has been known for >3 decades. The mechanisms underlying this disease, however, are just emerging. Several cellular and immunological mechanisms have been proposed to explain the pathogenesis of this disease. Recently, the development of animal models of CHB and the use of electrophysiological techniques to study the cellular and ionic mechanisms of maternal antibodies in heart cells provided new directions and alternative approaches for the pathogenesis of CHB. Garcia et al demonstrated that the IgG fraction of SSA/Ro and SSB/La antibodies induced abnormal conduction and reduction of Ca currents in rabbit heart. Subsequently, our laboratory demonstrated the arrhythmogenic effect of maternal autoantibodies in Langendorff-perfused hearts and further correlated these effects with the inhibition of the L-type Ca channel in...
isolated cardiac myocytes. In addition and unexpectedly, we reported significant sinus bradycardia in mice pups born to mothers injected with human maternal antibodies. These same maternal antibodies, however, did not affect the Na current ($I_{Na}$), the transient outward current ($I_{to}$), and the inward rectifier K current ($I_K$) in rat ventricular myocytes.

The present study was designed to address the following 3 questions: (1) Does inhibition of L-type Ca channels by maternal antibodies occur by direct interaction of the antibody with the channel pore-forming subunit? (2) Does maternal antibody affect T-type Ca channels (because this channel could be involved in the pacemaker activity of the heart)? (3) Does maternal antibody affect other channels, such as $I_{Na}$ and delayed rectifier $I_K$ channels. *Xenopus* oocytes were used to individually express these currents. This is most relevant for L-type and T-type Ca channels because of the unique advantage of separating T-type from L-type current, which is usually difficult to achieve in native cardiocytes.

**Methods**

**IgG Purification**

Purification of IgG has been performed as previously described. Briefly, immunoglobulin fractions containing IgG were purified from serum by protein A–Sepharose columns and confirmed to be pure by electrophoresis. IgGs were obtained from 3 mothers whose children have CHB. These IgGs were referred to as positive IgG and contain IgG against 48-kDa SSB/La, 52-kDa SSA/Ro, and 60-kDa SSA/Ro, as tested by ELISA and immunoblot. Negative IgG (control IgG) was purified from sera of 3 healthy mothers with healthy children and tested negative for anti-SSA/Ro and anti-SSB/La antibodies by ELISA and immunoblot. This dose-response relationship for the inhibition of $I_{Na}$ by positive IgG showed that the concentration of positive IgG that produced a half-maximal response ($EC_{50}$) was 173.5 ng/mL and that maximal inhibition was 350 µg/mL. Thus, an IgG concentration of 350 µg/mL was used throughout.

**Preparation of Xenopus Oocyte and cRNA Injection**

Mature female *Xenopus* frogs, purchased from Xenopus I (Ann Arbor, Mich), were anesthetized with 1.5 mg/mL tricaine. Surgically removed ovarian lobes were dissected and treated for 1.5 hours with 1.5 mg/mL collagenase type IA dissolved in Ca-free ND96 medium (mmol/L: NaCl 96, KCl 2, MgCl$_2$ 2, HEPES 5, pH 7.4). Stage IV and V oocytes were selected. cRNAs encoding the full length of the relevant for L-type and T-type Ca channels because of the

**Data Analysis**

Data acquired were stored, then analyzed offline with Pclamp 6 software (Axon Instrument Inc). All values are measured as the difference between zero and the peak current. The Microcal Origin v5.0 (Microcal Software Inc) program was used to generate figures and perform statistical analysis. Data are presented as mean±SEM. Student’s $t$-test for paired data and independent $t$-test or ANOVA was used when appropriate. A value of $P<0.05$ was considered statistically significant.
Results

L-Type I_{Ba} Was Inhibited by Positive IgG

Figure 1 shows the inhibitory effect of positive IgG on expressed L-type I_{Ba} in *Xenopus* oocytes. Figure 1A and 1B illustrates the I-V relations for I_{h}, I_{Ca} + I_{Ba} + I_{Na}, and I_{Ca} + I_{Ba} + I_{Na} before and after the addition of positive IgG. Positive IgG (350 μg/mL) inhibited I_{Ca} + I_{Ba} + I_{Na} by 50.9±4.2% (P<0.01, n=18) and 50.6±4.7% (P<0.01, n=12), respectively. Figure 1C shows the time course inhibition of I_{Ca} + I_{Ba} + I_{Na} by positive IgG in 1 typical oocyte. Application of positive IgG (350 μg/mL) resulted in 51% inhibition of I_{Ca} + I_{Ba} + I_{Na}. The effects of positive IgG were only partially reversible (86% recovery) before and after the addition of positive IgG. Positive IgG (350 μg/mL) on I-V relations of I_{Ca} + I_{Ba} + I_{Na} at 30 mV (P<0.01, n=10). The effects of positive IgG were not completely reversible (88% recovery after washing). Negative IgG did not significantly affect I_{Ca} + I_{Ba} + I_{Na} (Figure 1D). The average statistical data are summarized in the Table.

T-Type I_{Ba} Was Inhibited by Positive IgG

Figure 3 shows I_{h}, I_{f} in the absence and presence of IgG. Panel A illustrates the inhibitory effects of positive IgG on the I-V relations of I_{h}, I_{f}. Panel B shows the time course inhibition of I_{h} by positive IgG. Application of 350 μg/mL positive IgG resulted in 18.9±1.0% inhibition of I_{h} at -30 mV (P<0.01, n=10). The effects of positive IgG were not completely reversible (88% recovery after washing). Negative IgG did not significantly affect I_{h} (Figure 3C). The average statistical data are summarized in the Table.
Summary of the Effects of Positive IgG and Negative IgG on Expressed Currents

<table>
<thead>
<tr>
<th>IgG Applied</th>
<th>Subunit(s) Expressed</th>
<th>Control $I_{Ba}$, nA</th>
<th>Atropine, $I_{Ba}$, nA</th>
<th>acetylcholine, $I_{Ba}$, nA</th>
<th>$I_{Ba}$ After Addition of IgG, nA</th>
<th>% Decrease by IgG</th>
<th>n</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive IgG</td>
<td>$\alpha_{1C} + \beta_{3a} + \gamma_{2\delta}$</td>
<td>671.1±255.3</td>
<td>329.4±73.6</td>
<td>50.9±4.2</td>
<td>18</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive IgG</td>
<td>$\alpha_{1C}$</td>
<td>32.8±2.5</td>
<td>16.2±2.9</td>
<td>50.6±4.7</td>
<td>12</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive IgG</td>
<td>$\alpha_{1C} + \beta_{3a} + \gamma_{2\delta}$</td>
<td>522.5±162.3</td>
<td>274.1±120.6</td>
<td>47.5±4.6</td>
<td>7</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive IgG</td>
<td>$\alpha_{1C}$</td>
<td>37.9±9.1</td>
<td>18.0±4.7</td>
<td>47.4±3.5</td>
<td>5</td>
<td>0.016</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive IgG</td>
<td>$\alpha_{1C} + \beta_{3a} + \gamma_{2\delta}$</td>
<td>290±63.9</td>
<td>285±64.8</td>
<td>48.2±3.0</td>
<td>5</td>
<td>0.023</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive IgG</td>
<td>$\alpha_{1C}$</td>
<td>1099.5±230.2</td>
<td>892.0±189.0</td>
<td>18.9±1.0</td>
<td>10</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative IgG</td>
<td>$\alpha_{1C} + \beta_{3a} + \gamma_{2\delta}$</td>
<td>787.5±243.0</td>
<td>782.4±240.4</td>
<td>0.2±1.5</td>
<td>6</td>
<td>0.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative IgG</td>
<td>$\alpha_{1C}$</td>
<td>2305.0±146.0</td>
<td>2240.0±87.3</td>
<td>1.2±2.3</td>
<td>6</td>
<td>0.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive IgG</td>
<td>$\minK+\alpha_{1C}$</td>
<td>3604±326</td>
<td>3520±392</td>
<td>2.3±0.5</td>
<td>8</td>
<td>0.066</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive IgG</td>
<td>$\minK+\alpha_{1C}$</td>
<td>901.4±148.9</td>
<td>887.1±148.6</td>
<td>1.9±1.3</td>
<td>7</td>
<td>0.058</td>
<td></td>
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</tbody>
</table>

*P values before and after application of IgG.
†The percent decreases of L-type $I_{Ba}$ by positive IgG are not significantly different between $\alpha_{1C} + \beta_{3a} + \gamma_{2\delta}$ and $\alpha_{1C}$ groups (P=0.52).
‡The percent decreases of L-type $I_{Ba}$ by positive IgG are not significantly different between the groups treated with or without atropine or acetylcholine.
§The amplitudes of the currents before and after the application of atropine or acetylcholine are not significantly different. All current values represent peak current at 20 mV for L-type current, −30 mV for T-type current, −30 mV for Na current, and 40 mV for K current.

Positive IgG Did Not Affect Na ($I_{Na}$-hH1) and K ($I_{Ks}$-minK+KvLQT1) Channels

To check whether positive IgG affected other currents, we expressed Na current, $I_{Na}$-hH1, and K current, $I_{Ks}$-minK+KvLQT1, in oocytes. Figure 4 shows the effect of positive IgG on $I_{Na}$-hH1 (A) and $I_{Ks}$-minK+KvLQT1 (B). Positive IgG failed to significantly alter $I_{Na}$-hH1 (P=0.07, n=8) and $I_{Ks}$-minK+KvLQT1 (P=0.06, n=7).

Positive IgG Cross-Reacted With L-Type Ca Channel $\alpha_{1C}$-Subunit

To unambiguously demonstrate a direct interaction of positive IgG with Ca channel $\alpha_{1C}$ protein, we immunoprecipitated $\alpha_{1C}$ subunit from membranes of oocytes injected with $\alpha_{1C}$ subunit cRNA. A representative Western blot of 6 experiments is shown in Figure 5. Lane 1 shows Card I used as positive control, lane 2 positive IgG, lane 3 negative IgG, and lane 4 Card I after the blots of lane 3 were stripped. The $\alpha_{1C}$ subunit was detected as a band migrating above 200 kDa by Card I, as previously reported,22 and positive IgG but not negative IgG. This provides evidence that positive IgG directly cross-reacts with the $\alpha_{1C}$ subunit. Western blot experiments for T-type $\alpha_{1H}$ were not performed because antibodies against T-type Ca channel protein are not yet available.

Figure 3. Effects of positive and negative IgG on expressed $I_{Ba}$, recorded from Xenopus oocytes. A, Steady-state effects of positive IgG (350 μg/mL) on I-V relations of $I_{Ba}$ (n=6). B, Time course of inhibitory effects of positive IgG on $I_{Ba}$ in a different oocyte. C, Effects of negative IgG (350 μg/mL) on $I_{Ba}$ in a different oocyte (n=6). Insets, Selected current tracings at a test pulse of −30 mV from a single oocyte each, during control conditions and after application of IgG.

Figure 4. Effect of positive IgG on expressed $I_{Na}$-hH1 and $I_{Ks}$-minK+KvLQT1. A and B, Effect of positive IgG on $I_{Na}$-hH1 and $I_{Ks}$-minK+KvLQT1, respectively. Current tracings were obtained from a single oocyte each at a test pulse of −30 mV (holding potential −130 mV) for $I_{Na}$-hH1 and 0 mV and 40 mV (holding potential −60 mV) for $I_{Ks}$-minK+KvLQT1 during control conditions and after application of positive IgG. Similar results were found in 7 additional oocytes for $I_{Na}$-hH1 and 6 oocytes for $I_{Ks}$-minK+KvLQT1.
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Figure 5. Cross-reactivity of positive IgG with L-type Ca channel α1C protein. SDS-PAGE analysis of α1C protein immunopre-

cipitated with Card I antibody from oocytes injected with L-type Ca channel α1C subunit cRNA. Lane 1 shows reactivity to Card I, lane 2 to positive IgG, and lane 3 to negative IgG. Lane 4 shows reactivity to Card I after blot of lane 3 was stripped (this was done to demonstrate that α1C protein was present when subject-

ted to negative IgG). Similar results were found in 5 additional experi-

ments.

Discussion

The results presented here demonstrate that (1) positive IgG containing anti-SSA/Ro and anti-SSB/La antibodies from mothers whose children have CHB inhibited both L-type and T-type Ca channels but did not affect Na and K channels expressed in Xenopus oocytes; (2) the inhibitory effect of positive IgG was more marked (~51%) on L-type Ca channels than T-type Ca channels (~19%); (3) positive IgG inhibited $I_{\text{Ca}}$-α1C and $I_{\text{Ca}}$-α1C+β 2α+β 2β to a similar extent; (4) pretreatment of oocytes with atropine or acetylcholine did not alter the inhibition of expressed L-type $I_{\text{Na}}$ by positive IgG; (5) negative IgG did not affect either L- or T-type $I_{\text{Na}}$; and (6) immunoblot data unequivocally showed direct inter-

action between positive IgG and L-type Ca channel α1C subunit.

Maternal Antibody Inhibition of Expressed L-Type Ca Channels

The present findings that positive IgG, but not negative IgG, functionally inhibits Ca channels expressed in Xenopus oocytes are consistent with previous data from our laboratory in cardiac myocytes. Although we do not exclude the possibility that other endogenous auxiliary subunits, such as β-subunits, may be functionally associated with the expressed α1-subunit, our Western blot experiments unambiguously demonstrate that maternal autoantibodies directly cross-react with the pore-forming α1C-subunit (Figure 5). The α1C subunit migrated at ~200 kDa, and its size was similar to that in oocytes reported by others.

Conversely, the inhibition of $I_{\text{Na}}$ was not affected by pretreatment with atropine (a muscarinic receptor blocker). Bacman et al. reported that IgG present in the sera of patients with CHB and their mothers could bind and activate muscarinic cholinergic receptors of neonatal rat atrial prep-

arations. This raises the possibility that the inhibitory effect of positive IgG on $I_{\text{Na}}$ may be, at least in part, due to the activation of muscarinic receptors. Using an oocyte expres-

sion system, we did not find any difference in the inhibition of the expressed $I_{\text{Na}}$ by positive IgG in the absence and presence of atropine, suggesting either that the inhibitory effect of positive IgG does not involve muscarinic receptors or, alternatively, that oocyte muscarinic receptors are not coupled with expressed Ca channels. The present oocyte experiments do not rule out the possible regulation of Ca channels by positive IgG through normally coupled sarcolemmal receptors in native cardiocytes.

Maternal Antibody Inhibition of Expressed T-Type Ca Channels

Our data showed that maternal antibody blocks not only L-type Ca channels but also T-type Ca channels. Because T-type Ca channels have been implicated in the pacemaker activity in the heart, these findings may provide, at least in part, an ionic basis for the sinus bradycardia reported in murine models of CHB. This is further supported by in vivo data in conscious rats and in anesthetized dogs demonstrating a decrease in heart rate by mibebradil. Similar dose-dependent decreases in heart rate have been reported in humans. These novel findings are of clinical importance because it is only recently that clinicians caring for infants with CHB have begun focusing their attention on sinus bradycardia in addition to atrioventricular (AV) node conduction abnormalities. In this regard, Brucato et al. confirmed the sinus bradycardia we reported in the murine model in infants born to mothers seropositive to SSA/Ro antibodies.

Maternal Antibody Did Not Affect Na and K Channels

Positive IgG failed to affect expressed $I_{\text{Na}}$, $I_{\text{K}r}$, and $I_{\text{minK}}$. These findings are consistent with those obtained in native cardiac myocytes showing lack of effect of positive IgG on fast $I_{\text{Na}}$, $I_{\text{K}r}$, and $I_{\text{minK}}$. Furthermore, the lack of effect on these channels suggests that positive IgG preferentially interacts with Ca channels.

Pathogenesis of CHB

Available autopsies from affected infants showed the existence of myocarditis and fibrosis of the AV node. Because circulating maternal autoantibodies are directed against intracellular autoantigens, hypotheses have been proposed that intracellular SSA/Ro and SSA/La proteins are being trafficked to the cell surface during development by the induction of stress proteins, hormonal influences, viral infection, or apoptosis. The mechanisms by which these events alter AV conduction in fetal heart remain unclear.

It is only recently that electrophysiological and functional data proposed alternative explanations for CHB pathogenesis. Active and passive animal models for CHB have been established. Immunized pregnant mice gave birth to pups with complete AV block and significant sinus bradycardia. Furthermore, positive IgG induced AV block and bradycardia in acutely perfused isolated hearts and inhibited L-type Ca current from isolated cardiac myocytes. These findings suggest that apparent pathological changes, such as inflammation, are not necessarily a primary event for this disease and that the autopsy evidence may represent an advanced stage of the maternal antibody blockade of Ca channels, which play a vital role in the excitation-contraction coupling of the developing heart.

Consequences of L- and T-Type Ca Channel Blockade by Maternal Antibodies

L-type Ca channels are widespread in the cardiovascular system and are crucial in action potential propagation, con-
duction in the AV node, and excitation-contraction coupling in the heart. Blockade of L-type Ca channels by positive IgG coincides with the conduction block at the AV node and with the clinical finding that infants with CHB often have diminished ventricular function and heart failure.\textsuperscript{29,34} The function of the T-type Ca channel is less clearly defined, but it is thought to be involved in pacemaker activity in the heart.\textsuperscript{23}

L-type Ca channel density is lower\textsuperscript{35} and sarcoplasmic reticulum is less abundant\textsuperscript{36} in fetal heart cells than in adult cardiac cells. Thus, blockade of Ca channels by autoantibodies will impose a further burden on those marginally functioning Ca channels. It is also possible that prolonged and chronic exposure of fetal cardiac Ca channels to maternal antibodies could result in downregulation of the channels by internalization, leading to cell death, further exposing the intracellular SSA/Ro and SSB/La antigens to the circulating autoantibodies and ultimately resulting in inflammation, fibrosis, and at later stages, calcification. Thus, it is possible that the pathogenic activity of these autoantibodies may be primarily through Ca channel blockade and that the SSA/Ro and SSB/La ribonucleoproteins contribute as a secondary mechanism. Taken together, the present findings and those from several previous reports\textsuperscript{10,12,13} make the direct interaction of positive IgG with Ca channels an attractive hypothesis that could account, at least in part, for the pathogenesis of CHB.

Acknowledgments

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References

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