Functional Basis of Sinus Bradycardia in Congenital Heart Block

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Abstract—Congenital heart block (CHB) is a conduction abnormality characterized by complete atrioventricular (AV) block. CHB affects fetuses and/or newborn of mothers with autoantibodies reactive with ribonucleoproteins 48-kDa SSB/La, 52-kDa SSA/Ro, and 60-kDa SSA/Ro. We recently established animal models of CHB and reported, for the first time, significant sinus bradycardia preceding AV block. This unexpected observation implies that the spectrum of conduction abnormalities extends beyond the AV node to also affect the SA node. To test this hypothesis, we investigated the functional basis of this sinus bradycardia by characterizing the effects of antibodies from mothers with CHB children (positive IgG) on ionic currents that are known to significantly contribute to spontaneous pacing in SA node cells. We recorded L- (I_{Ca,L}) and T- (I_{Ca,T}) type Ca^{2+}, delayed rectifier K^+ (I_K), hyperpolarization-activated (I_H) currents, and action potentials (APs) from young rabbit SA node cells. We demonstrated that positive IgG significantly inhibited both I_{Ca,T} and I_{Ca,L} and induced sinus bradycardia but did not affect I_H and I_K. Normal IgG from mothers with healthy children did not affect all the currents studied and APs. These results establish that IgG from mothers with CHB children causes substantial inhibition of I_{Ca,T} and I_{Ca,L}, two important pacemaker currents in rabbit SA node cells and point to both I_{Ca,T} and I_{Ca,L} as major players in the ionic mechanism by which maternal antibodies induce sinus bradycardia in CHB. These novel findings have important clinical significance and suggest that sinus bradycardia may be a potential marker in the detection and prevention of CHB. The full text of this article is available online at http://circres.ahajournals.org. (Circ Res. 2004;94:e32-e38.)

Key Words: L-type calcium current ■ T-type calcium current ■ antibodies ■ congenital heart block

Congenital heart block (CHB), detected at or before birth in a structurally normal heart, is strongly associated with autoantibodies reactive with the intracellular soluble ribonucleoproteins 48-kDa SSB/La, 52-kDa SSA/Ro, and 60-kDa SSA/Ro. CHB is presumed to be due to the transplacental passage of these IgG autoantibodies from the mother into the fetal circulation. In addition to various degree of atrioventricular (AV) block, other neonatal abnormalities affecting the skin, liver, and blood elements are also associated with anti-SSA/Ro and -SSB/La antibodies in the maternal and fetal circulation and are grouped under the heading of neonatal lupus syndromes. To date, complete AV block is irreversible, although varying degrees of block have been noted, and second degree block has on rare occasion reverted to normal sinus rhythm.

We have recently reported that perfusion of Langendorff perfused rabbit hearts with IgG from mothers with CHB children (positive IgG) caused sinus bradycardia preceding AV block using surface ECG and optical action potentials. A significant and unexpected sinus bradycardia was also observed in the animal models of CHB developed by either passive transfer of positive IgG into pregnant mice or by active immunization of female mice or rabbits with SSA/Ro antigen. This high incidence of sinus bradycardia both in vitro and in vivo suggests the possible involvement of the sinusatrial (SA) node. Indeed, Brucato et al found sinus bradycardia in infants born to mothers seropositive to SSA/Ro antibodies. Based on these animal and clinical data, we hypothesized that positive IgG may affect SA node ion currents underlying the pacemaker, thus providing a functional explanation for this sinus bradycardia. Pacemaker activity in SA node cells is known to be due to a complex interplay of various ionic currents. Among these currents, L-type Ca^{2+} current, I_{Ca,L}, plays a significant role in both the diastolic depolarization and upstroke phase. Delayed rectifier K^+ current, I_K, is also important in diastolic depolarization. Hyperpolarization-activated inward current, I_H, and T-type Ca^{2+} current, I_{Ca,T}, are operative in early and late phases of the diastolic depolarization, respectively. In addition, changes in the time-independent currents can also affect the electrical activity in SA node. In the present study, we focused on the major time-dependent pacemaker currents to understand the negative chronotropic mechanism of autoantibodies from mothers with CHB children.
**Materials and Methods**

**Cell Isolation**
All experiments were performed in accordance with animal studies subcommittee regulations at VA New York Harbor Healthcare System. The isolation of single SA node cells was performed by the chopping method\(^1\) with a slight modification. New Zealand young rabbits weighing 0.9 to 1.5 kg were anesthetized with intravenous injection of pentobarbital sodium (40 mg/kg). The heart was rapidly excised and immersed in a normal Tyrode’s solution containing the following (in mmol/L): 140 NaCl, 5.4 KCl, 1.0 MgCl\(_2\), 1.8 CaCl\(_2\), 0.33 Na\(_2\)HPO\(_4\), 10 glucose, and 5 HEPES (pH 7.4). The SA node region was excised from the heart and strips about 0.5 to 1 mm wide were cut perpendicularly to the crista terminalis border. The strips were first incubated in an oxygenated Ca\(^{2+}\)-free Tyrode’s solution for 15 minutes, then in the Ca\(^{2+}\)-free Tyrode’s solution containing elastase (0.2 mg/mL, Boehringer Mannheim) and collagenase (85 U/mL, Worthington) for 45 to 60 minutes at 37°C. The cells were then dispersed by gentle trituration and were stored at 4°C. The cells were then incubated in elastase (0.2 mg/mL, Boehringer Mannheim) and collagenase (85 U/mL, Worthington) for 45 to 60 minutes at 37°C. The cells were then dispersed by gentle trituration and were stored at 4°C. The cells were then dispensed as single cells and used in this study.

**Solutions**
At the beginning of each experiment, all cells were first superfused with normal Tyrode’s solution and then switched to the appropriate solution for each current to be studied. Unless otherwise indicated, the standard pipette solution contained (in mmol/L): K glutamate 70, KCl 30, KH\(_2\)PO\(_4\) 10, MgCl\(_2\), 1; taurine 20, glucose 10, and HEPES 10. The cells used in this study were visually identified as long spindle-shaped cells and showed faint striation and prominent centrally located nuclei.

**Membrane Currents**
Membrane currents were recorded with amphotericin-perforated patch clamp techniques.\(^12\) Amphotericin B (6 mg) was dissolved in 100 µL dimethyl sulfoxide, from which 10 µL was added to a 3-mL pipette solution. The perforated patches were usually established within 10 minutes. The amphotericin-perforated patch recordings were used to reduce dilution of intracellular components, a possible cause of rundown of some membrane currents.

**Data**
Data were sampled with an A/D converter (Digital 1200, Axon Instruments) and stored on the hard disk of a computer for subsequent analysis. A programmable horizontal puller (Model P-87, Sutter Instrument Company) was used to pull the electrodes. Borosilicate glass electrode (outer diameter, 1.5 mm) with resistances of 2 to 5 MΩ when filled were connected to a patch-clamp amplifier (Dagan 3900A, Dagan Corporation). Junction potentials were zeroed before the pipette touched the cell and always compensated. Pipette series resistance was compensated to minimize the duration of the capacitative transient on 10-nA depolarization from −80 mV. The I\(_{\text{Ca,L}}\) was activated by depolarization pulses from a holding potential of −80 to −40 mV followed by 300-ms depolarization to 10 mV used to inactivate the fast Na\(^+\) current and I\(_{\text{K,ATP}}\) and minimize the rundown of I\(_{\text{Ca,L}}\). I\(_{\text{Ca,T}}\) was elicited by depolarizing pulses from a holding potential of −80 mV. A 1000-ms depolarization pulse from a holding potential of −40 mV to the test potentials was used to record I\(_{\text{K,ATP}}\) and was elicited on hyperpolarizations from a holding potential of −40 to −110 mV.

**Action Potential Recordings**
Action potentials (APs) were recorded during stable spontaneous electrical activity (current clamp conditions) using the same set-up for current recordings described above. Cells were superfused with Tyrode’s solution containing the following (in mmol/L): NaCl 140.0, KCl 5.4, Na\(_2\)HPO\(_4\) 0.33, CaCl\(_2\) 1.8, MgCl\(_2\) 0.5, glucose 5.5, and HEPES 5.0; pH was adjusted to 7.4 with NaOH. The pipette solution contained the following (in mmol/L): aspartic acid 100.0, KCl 30.0, MgCl\(_2\) 0.5, ATP- Na\(_2\) 5.0, GTP-Na\(_2\) 0.1, EGTA 11.0, and HEPES 10.0; CaCl\(_2\) 5.0 (pH was adjusted to 7.2 with KOH). Experiments were performed at 35±0.5°C.

**Data Analysis**
All results are presented as mean±SEM. Current density expressed as pA/pF was determined by dividing current amplitude with cell capacitance. Statistical significance was determined by a Student’s t test for paired data. A value of P≤0.05 was considered significant.

**Results**

**Effects of Positive IgG on I\(_{\text{Ca,L}}\) and I\(_{\text{Ca,T}}\)**
To determine the relative role of ionic currents affected by maternal antibodies from mothers with CHB children in single SA node cells, first, the effect of positive IgG (10, 50, 100, and 200 µg/mL) on peak I\(_{\text{Ca,L}}\) was examined. The dose-response curve of positive IgG on I\(_{\text{Ca,L}}\) yielded an IC\(_{50}\) of 59.4 µg/mL (Figure 1A). Figure 1B shows the time course of the time course of a representative current recording elicited by 300 ms depolarizing pulses to 10 mV from a holding potential of −40 mV (activating I\(_{\text{Ca,L}}\) preferentially over I\(_{\text{Ca,T}}\)) in a K\(^+\) free, Cs\(^+\)-containing solution in the presence of 5 mmol/L 4-AP before, during application of, and after washout of positive IgG. Application of positive IgG (100 µg/mL) reduced the peak of I\(_{\text{Ca,L}}\) from 191 to 112 pA (41.4%). The inhibition was partly reversed on washout of IgG (160.0 pA, 83.7% recovery). In the contrary, negative IgG (100 µg/mL) did not significantly alter the time course of I\(_{\text{Ca,L}}\). Figure 1C shows current density-voltage (I-V) relations of I\(_{\text{Ca,L}}\) during control and positive IgG (100 µg/mL). Positive IgG, but not negative IgG, significantly reduced I\(_{\text{Ca,L}}\) at voltages between 0 and +30 mV (Figure 1C: 46.2%±5.6% at 10 mV, n=5; P<0.01) without a significant shift in the steady state activation curve.
Positive IgG (500 μg/mL) did not have significant effect on $I_{K}$ at all potentials. The average inhibition of $I_{KL}$ at $-40$ mV before (control) and during application of positive IgG (100 μg/mL) was $5.2\%$ at $500 \mu g/mL$. Original traces for control, positive IgG, and washout are shown in the inset. C, Averaged current density-voltage relations of peak $I_{KL}$ in response to various depolarizing pulses from a holding potential of $-40$ mV before (control) and during application of positive IgG (100 μg/mL). *$P<0.05$; **$P<0.01$. D, Voltage-dependent activation properties of $I_{KL}$ before (control, $n=5$) and after positive IgG (n=5). Normalized values were fitted to Boltzmann equation ($\theta_{50}=1/(1+e^{(V_{1/2}-V)/k})$) to obtain the midpoint ($V_{1/2}$) and slope factor ($k$). $V_{1/2}=3.9\pm0.8$ mV, $k=8.9\pm0.7$ for control, and $V_{1/2}=5.4\pm0.9$ mV, $k=12.1\pm0.9$ for positive IgG.

**Figure 1.** Effect of positive IgG on $I_{KL}$. A, Dose-response relation for the effect of positive IgG on $I_{KL}$ at 10 mV from a holding potential of $-40$ mV. Curve was fit by non-linear regression to a sigmoidal function of the following form: effect=1/(1+($K_d/C$)^n), where $C$ is concentration, $K_d$ ($I_{KL}$) is the concentration for half-maximal effect, and $n$ is a constant. Each point represents mean±SEM data for 4, 5, or 6 cells at each positive IgG concentration, as indicated. B, Time course of peak $I_{KL}$ at 10 mV from single SA node cells before, during application of positive IgG, and after washout of positive IgG (100 μg/mL). Original traces for control, positive IgG, and washout are shown in the inset. C, Averaged current density-voltage relations of peak $I_{KL}$ in response to various depolarizing pulses from a holding potential of $-40$ mV before (control) and during application of positive IgG (100 μg/mL). *$P<0.05$; **$P<0.01$. D, Voltage-dependent activation properties of $I_{KL}$ before (control, $n=5$) and after positive IgG (n=5). Normalized values were fitted to Boltzmann equation ($\theta_{50}=1/(1+e^{(V_{1/2}-V)/k})$) to obtain the midpoint ($V_{1/2}$) and slope factor ($k$). $V_{1/2}=3.9\pm0.8$ mV, $k=8.9\pm0.7$ for control, and $V_{1/2}=5.4\pm0.9$ mV, $k=12.1\pm0.9$ for positive IgG.
bradycardia at irregular firing intervals (about 78 bpm, Figure 5B). After 2 minutes of superfusion with positive IgG, further bradycardia (about 66 bpm) was observed (Figure 5C). After 10 minutes superfusion with Tyrode’s solution, only partial recovery was seen (Figure 5D). During positive IgG application, the AP amplitude was reduced (from 84.3 ± 7.9 to 71.5 ± 12.6 mV; P < 0.05, n = 5), the slope of phase 4 was also reduced (from 62.8 ± 4.2 to 41.9 ± 6.9 mV/sec; P < 0.05, n = 5) without significant change in the maximum diastolic potential (MDP; control − 63.8 ± 2.6 mV versus − 58.0 ± 7.0 mV; P = NS, n = 5). In contrast, superfusion of SA node myocytes (n = 3) with negative IgG did not alter the spontaneous AP rate, AP amplitude, phase 4 slope, or MDP (AP rate, 164.3 ± 10.5 versus 162.5 ± 12.5 bpm; AP amplitude, 85.5 ± 8.6 versus 86.3 ± 9.6 mV; phase 4 slope, 61.5 ± 5.5 to 62.4 ± 9.6 mV/sec; P = NS, n = 5; MDP, − 62.5 ± 4.5 versus − 61.8 ± 6.6 mV, respectively).

**Discussion**

In the present study, we found that maternal antibodies from mothers of children with CHB decreased I_{Ca,L} and I_{Ca,T}, two important currents to spontaneous cardiac pacing, without altering I_K and I_f in rabbit SA node cells. In addition, positive IgG caused sinus bradycardia in single SA node myocytes. These effects were not seen with normal IgG from healthy mothers with healthy children. This is the first study that provides a functional basis for sinus bradycardia associated with CHB, and points to the important role of both I_{Ca,T} and I_{Ca,L} in this sinus bradycardia.
Bradycardia and SA Node Involvement

Because abnormalities of the AV node are the hallmark of autoantibody-associated CHB, the AV node rather than the SA node was the main focus of previous publications5–8 even during routine clinical diagnosis of CHB.9 Although Garcia et al,13 using isolated rabbit heart, and our group, using Langendorff perfused isolated rabbit5 and human fetal hearts,7 have also observed significant sinus bradycardia in their models, this bradycardia was not emphasized and its electrophysiological basis have not been investigated. Unexpectedly, we also observed high incidence of sinus bradycardia in an experimental mouse model of CHB developed by passive transfer of human autoantibodies into pregnant mice6 and by direct immunization of female mice or rabbits with SSA/Ro antigen,7,8 suggesting a possible involvement of SA node. This observation is further supported by clinical data by Brucato et al,9 demonstrating sinus bradycardia in children born to mothers seropositive to SSA/Ro antibodies. This novel finding is 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 AV node conduction abnormalities. Indeed, human fetal autopsies14,15 showed calcification of the SA node, further suggesting that the SA node may be affected. 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.16–19 The mechanisms by which these events alter SA node pacemaker activity remain unclear.

Effect of Positive IgG on the Membrane Currents and Action Potential in SA Node Cells

In general, the total IgG levels of CHB patients are higher than that from healthy individuals. The level of IgG in CHB cord sera at the time of delivery varied from 500 to 1500 mg/dL,20 which corresponds to 5 to 15 mg/mL. To study the role of antibodies from mothers with CHB children on currents involved in spontaneous pacing, we used the concentration of IgG (80 to 100 μg/mL), which we have previously shown to inhibit Ca channels in single ventricular myocytes7–21 and determined dose-response curves as shown.
in Figures 1A and 2A. Higher concentrations of 800 to 1200 μg/mL were required to induce complete AV block in whole human fetal and rabbit heart perfused in a Langendorff fashion. Because the serum specimens from the infants were obtained after birth, ie, weeks later after CHB manifestation in the fetus, the exact concentration of IgG at that time is not known.

Single SA node cells were voltage-clamped to assess the effect of positive IgG on the four major time-dependent ionic currents involved in diastolic depolarization, $I_N$, $I_{CaL}$, $I_{CaT}$, and $I_C$. Our data show that both $I_{CaL}$ and $I_{CaT}$ are reduced significantly by positive IgG in the single rabbit SA node cells. This is consistent with the observed inhibition of phase 4 diastolic depolarization in SA node cells, suggesting that the negative chronotropic effect of positive IgG is derived, at least in part, from reduction of both $I_{CaL}$ and $I_{CaT}$.

In the present study, we showed $I_{CaL}$ inhibition by positive IgG, suggesting that the L-type Ca$^{2+}$ channel is a target for maternal antibodies in SA node cells. The consequences of this inhibition may account for the sinus bradycardia. Because we show that positive IgG reduces $I_{CaL}$ without shifting $I-V$ relations or activation curve (voltage dependence was unchanged), the underlying mechanism for positive IgG-induced reduction of $I_{CaL}$ may not be due to changes in channel gating. Indeed, we have previously demonstrated that positive IgG inhibited ventricular $I_{CaL}$ by reducing open times and increasing closed times at the single channel level in both human fetal and rat heart. We proposed that these data may, in part, explain the basis of the whole-cell $I_{CaL}$ inhibition by positive IgG in the present study. However, the exact mechanism by which positive IgG affects $I_{CaL}$ gating in the rabbit SA node is yet to be determined.

T-type Ca$^{2+}$ channels are usually present in the SA cells and Purkinje fibers of the heart. The physiological role of the T-type Ca$^{2+}$ channel is not completely understood, and believed to be involved in the pacemaker activity. Indeed, in vivo studies have shown that sinus bradycardia can be induced in conscious rats and in anesthetized dogs by mibebradil alone, a selective T-type Ca current blocker. Similar dose-dependent decrease in heart rate has been also reported in human. Therefore, both $I_{CaL}$ and $I_{CaT}$ in SA node cells may contribute to the negative chronotropic effect of maternal antibodies to Ro/La.

It is noteworthy that normal IgG lacking anti-Ro/SSA and anti-La/SSB antibodies did not affect both Ca$^{2+}$ channels (L- and T-types), indicating that it is unlikely that other unidentified components of IgG may contribute to the effects. However, because we did not use affinity-purified antibodies in this study, we cannot completely rule out the contribution of unidentified components of IgG to our observations.

We have previously shown that positive IgG did not alter the transient outward K current, $I_{to}$, the inward rectifier K current, $I_K$, and the fast Na current, $I_{Na}$. Because time-dependent currents are absent after the administration of E-4031 plus nifedipine, the instantaneous $I-V$ relation represents a background current. This background current could be a mix of some time-independent current that have been suggested to be involved in SA node pacemaking activity, ie, Na$^+-$K$^+$ pump, Na$^+-$Ca$^{2+}$ exchanger. However, we did not see any effect on the net current after applying positive IgG, indicating that this time-independent background current may not be involved in bradyarrhythmia associated with CHB. Altogether, positive IgG seems to selectively affect Ca$^{2+}$ channels (L- and T-types) but not other currents such as $I_N$, $I_K$, $I_{Na}$, and $I_{to}$, suggesting specificity to Ca$^{2+}$ channels.

Potential Significance

In rabbit SA node cells, $I_{CaL}$ and $I_{CaT}$ are important currents in late phase of diastolic depolarization. In the present study, we found that both $I_{CaL}$ and $I_{CaT}$ were reduced by positive IgG in SA node cells. Our observations provide direct evidence for the ionic mechanism of the negative chronotropic action of maternal antibodies to SSA/Ro and SSB/La proteins associated with CHB. The findings raise the possibility that sinus bradycardia which often precedes AV block may indicate the potential for AV conduction abnormalities. The findings also provide new insights to the pathogenesis of CHB and potentially to the therapeutic management of a disease considered irreversible and for which currently available therapies are refractory.

Acknowledgments

This study was supported by an NIH grant (HL-55401) and VA Medical Research Funds (Merit Grant Award and REAP grant) to M.B. IgGs were kindly provided by Dr Jill Buyon through the Research Registry for Neonatal Lupus (AR-4220). We would like to thank the animal laboratory staff for their assistance.

References


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_Circ Res._ 2004;94:e32-e38; originally published online February 12, 2004;
doi: 10.1161/01.RES.0000121566.01778.06
_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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