Developmental Changes in Electrophysiological Properties and Synaptic Transmission in Rat Intracardiac Ganglion Neurons

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Rimmer, Katrina and Alexander A. Harper. Developmental changes in electrophysiological properties and synaptic transmission in rat intracardiac ganglion neurons. J Neurophysiol 95: 3543–3552, 2006. First published April 12, 2006; doi:10.1152/jn.01220.2005. We charted postnatal changes in the intrinsic electrophysiological properties and synaptic responses of rat intrinsic cardiac ganglion (ICG) neurons. We developed a whole-mount ganglion preparation of the excised right atrial ganglion plexus. Using intracellular recordings and nerve stimulation we tested the hypothesis that substantial transformation in the intrinsic electrophysiological characteristics and synaptic transmission accompany postnatal development. Membrane potential ($E_m$) did not change but time constant ($\tau$) and cell capacitance increased with postnatal development. Accordingly, input resistance ($R_{in}$) decreased but specific membrane resistance ($R_{m}$) increased postnatally. Comparison of the somatic active membrane properties revealed significant changes in electrical phenotype. All neonatal neurons had somatic action potentials (APs) with small overshoots and small afterhyperpolarizations (AHPs). Adult neurons had somatic APs with large overshoots and large AHP amplitudes. The range of AHP duration was larger in adults than in neonates. The AP characteristics of juvenile neurons resembled those of adults, with the exception of AHP duration, which fell midway between neonate and adult values. Phasic, multiply adapting, and tonic evoked discharge activities were recorded from ICG neurons. Most neurons displayed phasic discharge at each developmental stage. All neurons received excitatory synaptic inputs from the vagus or interganglionic nerve trunk(s), the strength of which did not change significantly with postnatal age. The changes in the electrophysiological properties of the postganglionic neuron suggest that increased complexity of parasympathetic regulation of cardiac function accompanies postnatal development.

INTRODUCTION

Intrinsic cardiac ganglia (ICG) are interconnected clusters of neurons located throughout the atrial epicardium and interatrial septum. They are innervated by the vagus nerve and send their projections to discrete regions of the heart. The final pattern of discharge in these neurons regulates chronotropic, dromotropic, and inotropic elements of cardiac function (Adams and Cuevas 2004).

Traditionally the intrinsic cardiac nervous system was held as a simple relay for parasympathetic inputs to cardiac end effectors. However, recent work has clearly indicated that in the adult these peripheral ganglia are complex neural networks with multiple neuronal subtypes and are capable of complex reflex control of regional cardiac function (Arvold 2004). Autonomic control of the heart changes during early postnatal development in mammals. Direct vagal nerve stimulation studies reveal significant decreases with postnatal age in the intrinsic heart rate and parasympathetic control of the heart (Gootman 2004).

In the rat, the parasympathetic response is established before birth, whereas functional sympathetic innervation of the heart is not present until 2 wk of age (Robinson 1996). Understanding the electrophysiological specialization of the neuronal elements and the strength and spatial distribution of interneuronal coupling within the living ganglion is a prerequisite to understanding its normal function. Appreciation of the innervation of ICG will lead to greater insight into the dysfunction and failure of this autonomic pathway, such as the diminished parasympathetic tone that is a hallmark of cardiovascular disease and aging (Lagi et al. 1999).

Both the electrical properties and synaptic responses of ICG neurons must be characterized to assess changes in neuronal integration within the intrinsic cardiac nervous system that occur during development. ICGs of adult animals contain a heterogeneous population of neurons, identified on the basis of their electrical and synaptic properties (Edwards et al. 1995; Selyanko 1992; Selyanko and Skok 1992). However, it is not known when the specification of neuronal subtypes occurs. The intrinsic properties of a neuron are not static, but can alter during development by the up- or downregulation of expression of ion channels. In studies using rat dissociated ICG neurons the expression and characteristics of the hyperpolarization-activated ($I_h$) and inward rectifier K$^+$ [$I_{K(IR)}$] currents (Hogg et al. 2001), ATP-sensitive K$^+$ conductance [$I_{K(ATP)}$] (Hogg and Adams 2001), and $\gamma$-aminobutyric acid type A (GABA_A) receptor channels (Fischer et al. 2005) all show changes during postnatal development.

There is little information regarding postnatal development of electrical characteristics in parasympathetic ganglia, although there is a reasonable cohort of data for sympathetic ganglia, where in many cases the functional phenotype changes with development. For example, Anderson et al. (2001) examined the pre- and postnatal electrophysiological properties of sympathetic neurons. This study concluded that much of the diversity of electrophysiological characteristics in the mature animal is acquired during fetal development.

Parasympathetic regulation of cardiac performance originates in the CNS and traverses the parasympathetic ganglia. Synaptic activation of the neurons in the cardiac ganglia thus needs to be considered as part of this pathway from the brain to the heart. Several animal models have been used for studying synaptic responses in the mature, adult ICG: guinea pigs (Edwards et al. 1995); dogs (Bibevski et al. 2000; Xi-Moy et...
al. 1993); pigs (Smith 1999); and rats, adult (Selyanko and Skok 1992) and neonatal (Seabrook et al. 1990). Nerve-evoked responses were blocked by hexamethonium and so are mediated by nicotinic acetylcholine receptors (Bibeviski et al. 2000; Selyanko and Skok 1992). However, in some neonatal rat ICG neurons synaptic transmission was resistant to blockade by the nicotinic antagonist mecamylamine, suggesting a noncholinergic component (Seabrook et al. 1990).

We developed a whole-mount rat cardiac ganglia preparation that allows electrical properties and synaptic responses of neurons to be analyzed. We studied three time points during postnatal development: neonates (2–5 days), juveniles (14–21 days), and adults (≥6 wk). The juvenile time window was chosen to coincide with the development of sympathetic control of cardiac regulation. The postnatal gap between parasympathetic and sympathetic development affords a valuable model for studying integration of chemical signaling within ganglia.

We hypothesize that generation of the several classes of neurons reported for the mature ICG occurs during postnatal development, as observed in dissociated ICG neurons (Hogg et al. 2001). However, results from dissociated neurons are not always in accordance with those from whole-mount preparations; for example, P2X receptor channels are not expressed in whole-mount postganglionic submandibular (Smith et al. 2001) and sympathetic (Inokuchi and McLachlan 1995) neurons but are present in dissociated neurons from these ganglia. If there are any alterations in electrophysiological characteristics and synaptic transmission, the next step is to determine whether they are simply associated with postnatal increases in neuronal size or whether they follow functional sympathetic innervation. Preliminary aspects of these results were previously reported (Rimmer and Harper 2004).

**METHODS**

**Preparation**

Wistar rats (Harlan UK, Oxon, UK) were used at three stages of postnatal development: neonates (P2–P5), juveniles (P14–P21), and young nonpregnant female adults (≥6 wk, 150–220 g). Rats were killed by stunning and cervical dislocation, in accordance with current UK Home Office guidelines. The heart and lungs were quickly excised and placed in ice-cold Krebs solution. The right atrial ganglion plexus and underlying myocardium were isolated from the dorsal surface of the atria. The whole-mount preparation was pinned out in the recording chamber (35-mm petri dish lined with Sylgard No. 184 [Dow-Corning, Midland, MI], approximately 1.0 ml volume) and superfused with bicarbonate-buffered Krebs solution (approximately 2 ml/min), gassed with 95% O2-5% CO2 to pH 7.4. The temperature was controlled by a Peltier heating device (Medical Instruments, Yellow Springs, OH). The tissue was left to resuscitate in contrast optics on a fixed-stage microscope. Recordings were normally made from the largest ganglion, located at the junction of the right ventricle cava and right atrium, which regulates the sinoatrial node (Sampaio et al. 2003).

**Electrophysiological recordings**

Intracellular current-clamp recordings were made from postganglionic ICG somata using sharp glass microelectrodes (GC120F; Harvard Apparatus, Edenbridge, UK) with resistances of approximately 120 MΩ when filled with 0.5 M KCl. Membrane voltage responses were recorded with a conventional bridge amplifier (Axoclamp 2A, Axon Instruments, Union City, CA). Voltage signals were filtered at 20 kHz (Frequency Devices 902), digitized at 50 kHz, and transferred to a Pentium 4 computer using an A/D converter (Micro 1401 MKII interface, CED, Cambridge, UK) and Spike2 software (CED).

Two pulse protocols were used: short depolarizing pulses (≤3 ms in duration) to directly evoke single action potentials and 200 ms long hyperpolarizing and depolarizing pulses to measure passive properties and discharge characteristics, respectively. For short pulses, action potential parameters measured were overshoot and AHP duration to 50 and 80% recovery (AHP50, AHP80), and AHP amplitude using a Spike2 script. Input resistance (Rin) was calculated from the steady state of the voltage response to small hyperpolarizing long-current pulses (≤−0.1 nA), and time constant (τ) was measured by fitting 20–80% of the rising phase at τ=100 pA with a single exponential function using Origin software (Microcal, version 6, Northampton, MA). Discharge activity was classified as being phasic, multiply adapting, or tonic on application of a current pulse at twice threshold intensity.

**Nerve stimulation**

Branches of the vagus and interganglionic nerve trunks were stimulated using a suction electrode connected to a constant-voltage isolated stimulator (Digitimer DS2, Herts, UK). Nerve trunks were stimulated using stimulus pulses of 0.02 to 0.2 ms width, 5 to 50 V amplitude.

**Solutions and pharmacological agents**

Krebs solution contained (in mM): 118 NaCl, 25 NaHCO3, 1.13 NaH2PO4, 4.7 KCl, 1.8 CaCl2, 1.3 MgCl2, glucose 11.1 (Smith et al. 2001), and all reagents were of analytical grade. Apamin was purchased from Alomone (Jerusalem, Israel).

Data are presented as the means ± SD of the number of observations indicated, and were compared using ANOVA (Tukey–Kramer multiple comparisons test), paired and unpaired t-tests, and χ2 tests (GraphPad InStat software, Version 3.00, GraphPad Software, San Diego, CA) as indicated in the text.

**RESULTS**

All results presented are from ICG neurons with a resting membrane potential of at least −40 mV and overshooting somatic action potentials. Recordings were stable for ≥10 min before taking readings.

Many neurons in the intact ICG exhibited spontaneous excitatory postsynaptic potential (EPSP) activity, and occasionally spontaneous action potentials (APs) were recorded that were overshooting. The EPSPs normally had amplitudes at least an order of magnitude greater than recording noise and had a fast rising phase and a falling phase similar to the time constant of the neuron. Such activity was prevalent in the neonatal ICG with nearly 60% of neurons (18/31) displaying spontaneous events, decreasing with postnatal age to nearly 53% (23/43) in juveniles and 33% (14/42) in adults. Cd2+ (100 μM) inhibited spontaneous EPSPs and APs in neurons of two juveniles, and one neonatal. In contrast to previous reports (Edwards et al. 1995; Selyanko 1992) no instances of spontaneous sustained rhythmical action potential discharge, akin to pacemaker-like activity, were recorded. Sustained repetitive discharge could be evoked in some neurons on injection of small depolarizing currents (data not shown).
Postnatal developmental changes in postganglionic membrane properties

PASSIVE PROPERTIES. Membrane potential ($E_m$) was similar: approximately $-45$ mV at the three postnatal stages sampled. Neurons with an $E_m$ of $>-60$ mV were normally inexcitable and were excluded from this analysis because it was unclear whether these were neurons or glial cells. These acceptance criteria may explain why the $E_m$ values reported in this study are slightly more positive compared with other data from adult rat ICG neurons (e.g., Selyanko 1992; Xi-Moy and Dun 1995). There were changes in input resistance ($R_{in}$) and time constant ($\tau$) of ICG neurons during postnatal development (Table 1 and Fig. 1B). In neonatal ICG neurons the input resistance ($R_{in}$) was significantly greater than that of juveniles and adults. Time constant ($\tau$) and calculated cell capacitance ($C_{cell}$) increased with age, mirroring the increase in neuron size (Fig. 1B). This is depicted in the photomicrographs shown in Fig. 1A. A decrease in input resistance would be expected to accompany increased cell size, as indicated by cell capacitance. Interestingly, the $R_{in}$ values for juvenile and adult ICG neurons were similar. Membrane resistance ($R_{m}$) values were calculated from $R_{m} \times$ cell capacitance (assuming 1 pF = $100 \mu$m$^2$) and increased significantly from neonates to adults (see Table 1). This is in accordance with a previous study of dissociated ICG neurons (Hogg et al. 2001).

ACTIVE PROPERTIES. The active properties of adult ICG neurons were distinct from those of neonatal neurons (Table 2). Representative action potentials evoked by short (+0.3 nA) current pulses, from a neonatal and two individual adult neurons, are shown in Fig. 2A. The AP overshoot (OS) in neonatal neurons (approximately 9 mV) was significantly less than that from juveniles and adults (approximately 18 mV). The AHP after the action potential was characterized by its amplitude and decay time. Action potentials in neonatal ICG neurons had small amplitude AHPs, ranging from 5.8 to 14.6 mV, with a mean value of 10.3 mV. Adult AHP amplitude was significantly greater compared with the neonatal AHP, with a mean value of 17.7 ± 3.1 mV (Fig. 2A and Table 2).

Several ways of measuring AHP duration have been described for ICG neurons, such as time to 50% recovery, AHP50, and AHP80 values at each stage of development (Fig. 2B) and Table 2). Recorded for ICG neurons, such as time to 50% recovery, AHP50, and AHP80 values at each stage of development (Fig. 2B) and Table 2). Recorded for ICG neurons, such as time to 50% recovery, AHP50, and AHP80 values at each stage of development (Fig. 2B) and Table 2).

Evoked discharge

To characterize firing discharge, 200 ms depolarizing current pulses, +0.1 to +0.5 nA, were applied. Neurons were split into three groupings on the basis of firing properties at twice threshold stimulus intensity: phasic, multiply adapting and tonic; an example of each is shown in Fig. 4. A, B, and C for neonates, juveniles, and adults, respectively. The number of

Neurons from ICG in neonates had short AHPs, for which the mean duration of AHP50 was 11.3 ms and the mean AHP80 was 25.0 ms. The corresponding maximum duration was <17.5 and <50 ms, respectively (Fig. 2B). Juvenile and adult neurons exhibited a greater range of AHP80 values: 16.2–139 and 15.3–163 ms, respectively. The incidence of neurons with AHP80 values >50 ms increases with development: 10/43 juvenile and 22/41 adult neurons had an AHP80 >50 ms. No neurons recorded possessed slow AHPs (I_{slow}), which have a duration of 1–2 s, reported first for myenteric neurons (reviewed in Sah and Faber 2002). Histograms were plotted of adult AHP50 and AHP80 values (Fig. 2C) to show the frequency distribution for each. Table 2 presents the mean AHP50 and AHP80 data for each stage of development. The mean values for juvenile AHP50 and AHP80 were approximately midway between that for neonates and adults (Fig. 2B).

Juvenile data were separated into sets: P14–P15, P16–P19, and P20–P21 days, to ascertain whether increased functional sympathetic development has any impact on active and passive membrane properties, because the sympathetic nervous system begins to appear at P14 and is fully functional by P21 (Robinson 1996). With the exception of AHP amplitude, there was no significant difference in any of the membrane properties measured, suggesting that active properties have attained near-adult values by P14.

We hypothesized that the increase in AHP50 and AHP80 durations results from increasing expression of small Ca$^{2+}$-activated K$^+$ channels (SKCa), whose current underlies the AHP (Sah and Faber 2002). To test this, 100 nM apamin—a selective SK channel blocker—was applied to four adult and four neonatal neurons (Fig. 3A). The AHP50 and AHP80 values in control conditions and in the presence of apamin are plotted in Fig. 3B. Apamin reduced the mean AHP50 values from 17.0 ± 2.3 to 11.6 ± 0.8 ms (P < 0.05) in neonates and from 31.5 ± 10.0 to 11.0 ms (P < 0.05) in adults. The AHP80 was also shortened: 43.2 ± 4.8 to 20.6 ± 2.7 ms (P < 0.001) in neonates and 62.7 ± 14.4 to 20.2 ± 2.1 ms in adults (P < 0.001, paired t-test). Thus apamin reduces the AHP to nearly the same duration in neonates and adults.

TABLE 1.  Postnatal changes in the passive electrical properties of ICG neurons

<table>
<thead>
<tr>
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<th>Neonate</th>
<th>Juvenile</th>
<th>Adult</th>
<th>P Values</th>
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<tbody>
<tr>
<td>$E_m$, mV</td>
<td>$-46.6 \pm 4.9$ (32)</td>
<td>$-44.7 \pm 4.0$ (43)</td>
<td>$-47.2 \pm 6.4$ (44)</td>
<td>ns</td>
</tr>
<tr>
<td>$R_{in}$, MΩ</td>
<td>$161.6 \pm 60.7$ (32)</td>
<td>$127.7 \pm 52.5$ (40)</td>
<td>$118.2 \pm 52.6$ (44)</td>
<td>n vs. a***</td>
</tr>
<tr>
<td>$\tau$, ms</td>
<td>$4.2 \pm 2.0$ (32)</td>
<td>$5.8 \pm 3.3$ (40)</td>
<td>$6.7 \pm 3.8$ (44)</td>
<td>n vs. j*</td>
</tr>
<tr>
<td>C, pF</td>
<td>$27.7 \pm 10.7$ (32)</td>
<td>$47.9 \pm 23.4$ (40)</td>
<td>$62.6 \pm 30.7$ (44)</td>
<td>n vs. a***</td>
</tr>
<tr>
<td>$R_{m}$, kΩ cm$^2$</td>
<td>$4.2 \pm 2.0$ (32)</td>
<td>$5.8 \pm 3.3$ (40)</td>
<td>$6.7 \pm 3.8$ (44)</td>
<td>n vs. j***</td>
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Values are means ± SD; number of neurons in parentheses. ANOVA was used to calculate P values: *P < 0.05; ** P < 0.01; and *** P < 0.005; ns, not significant. $R_{in}$ values calculated for current steps $-100$ pA or less. $R_{m}$ values calculated from $R_{m} \times$ cell capacitance, assuming 1 pF = $100 \mu$m$^2$.
action potentials fired at twice-threshold current intensity is plotted for each neuron in Fig. 4B: 77% of neonatal neurons were phasic, 10% multiply adapting, and 13% were tonic. Juvenile neurons exhibited phasic firing in 70% of observed cases; multiply adapting, 15%; and those remaining, 15% tonic. For adults, 76% were phasic neurons, 12% multiply adapting, and 12% tonic. Application of apamin (100 nM) changed the firing properties from phasic to tonic in the neonatal (n = 4) and adult (n = 3) neurons studied (data not shown).

Hyperpolarizing current pulses can induce time-dependent rectification (TDR), held as the signature of the H-current (Pape 1996). Such behavior was observed in most neurons at all stages of development. The extent of TDR was quantified by measuring steady-state voltage response to a hyperpolarizing current pulse to approximately −100 ± 10 mV and expressing this as a percentage of peak membrane potential excursion. The values were 96 ± 3% (n = 32), 94 ± 3% (n = 42), and 94 ± 3.2% (n = 44) for ICG neurons of neonates, juveniles, and adults, respectively. The adult and juvenile values were significantly different from those of neonates (P values of <0.01 and <0.05, respectively; ANOVA). Examples of voltage traces and current–voltage plots displaying the range of inward rectification from each developmental stage are shown in Fig. 5.

Synaptic responses

Electrical stimulation of branches of the vagus (preganglionic) or interganglionic nerve trunks was performed using rectangular constant voltage pulses (width 0.02–0.2 ms; 5–50 V). The stimulus duration and amplitude required to evoke a synaptic response were normally greater for neonates than for juvenile and adult ICG neurons. This presumably reflects the caliber and state of myelination of the efferent axons, because the final steps of association between axons and Schwann cells occur in late embryonic and early postnatal stages (Jessen and Mirsky 2005). Two stimulus protocols were used: single stimuli presented at frequencies of ≤0.2 Hz (Fig. 7) and trains of 20 twice-threshold intensity stimuli at 5, 10, 20, and 50 Hz with a 30 s intertrain interval (Fig. 8A). The latter protocol was also used to ascertain the following frequency for direct, intrasomatic stimulation and to correlate these data with those of synaptic transmission (Fig. 8B). Synaptically evoked postsynaptic events in adult (n = 5) and neonatal (n = 5) ganglia were reversibly blocked by nominally Ca²⁺ free, high Mg²⁺ (6 mM) Krebs solution (Fig. 6A), as previously demonstrated for adult rat intracardiac ganglia (Selyanko and Skok 1992). Superfusion of 0 Ca²⁺/high Mg²⁺ Krebs solution produced a dramatic depolarization (9.9 ± 6.3 mV, n = 5) in neurons from neonatal ganglia, and less so in adult neurons (3.6 ± 2.8 mV, n = 5).

In addition to preganglionic axons, it has been reported that the vagus and ICG nerve connectives contain postganglionic axons (Edwards et al. 1995). Stimulating the vagus may evoke APs in these axons that will be conducted antidromically to ICG neurons. In three instances putative antidromic APs were recorded, indicated by very short latencies, no apparent EPSP, and discharge of all-or-nothing somatic APs (Selyanko and Skok 1992). To test whether these action potentials were antidromic, the wide-spectrum Ca²⁺ channel blocker cadmium
MD DEVELOPMENTAL CHANGES IN INTRACARDIAC GANGLION NEURONS

TABLE 2.  Action potential and AHP properties of ICG neurons from neonatal, juvenile, and adult rats

<table>
<thead>
<tr>
<th></th>
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<th>Juvenile</th>
<th>Adult</th>
<th>P Values</th>
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<tr>
<td>APₘᵥ, mV</td>
<td>9.4 ± 5.6 (31)</td>
<td>17.9 ± 7.6 (43)</td>
<td>19.3 ± 7.1 (41)</td>
<td>n vs. a, j***</td>
</tr>
<tr>
<td>AHP, mV</td>
<td>10.4 ± 2.6 (31)</td>
<td>15.7 ± 3.0 (43)</td>
<td>17.7 ± 3.1 (41)</td>
<td>n vs. a, j***</td>
</tr>
<tr>
<td>AHPₕ₀, ms</td>
<td>11.2 ± 2.8 (31)</td>
<td>19.2 ± 2.8 (43)</td>
<td>31.3 ± 2.3 (41)</td>
<td>j vs. a***</td>
</tr>
<tr>
<td>AHPₕ₀, ms</td>
<td>25.0 ± 8.4 (31)</td>
<td>43.0 ± 23.9 (43)</td>
<td>60.6 ± 33.0 (41)</td>
<td>n vs. a***</td>
</tr>
</tbody>
</table>

Values are means ± SD; number of neurons in parentheses. ANOVA was used to calculate P values: ** P < 0.01 and *** P < 0.005.

(100 μM Cd²⁺), which blocks synaptic transmission, was added to the superfusing Krebs solution (Fig. 6B). Cd²⁺ did not block antidromic conduction, but did reduce AP overshoot and AHP amplitude and duration, presumably as a result of the failure of activation of Ca²⁺-activated K⁺ channels. *Eₘ* did not change after addition of Cd²⁺ in neurons of the three adults, two juveniles, and one neonatal studied (grouped data were added to the superfusing Krebs solution (Fig. 6C). In neonates, orthodromic responses were subjectively classified into three groups based on the synaptic response and AP waveform: Group 1: *weak*, where nerve stimulation evokes a subthreshold EPSP. Group 2: *secure*, where AP arises late in the EPSP when present or can occur without an underlying EPSP. Group 3: *strong*, where AP arises early during the EPSP. These synaptic responses are shown in Fig. 7A and the relative proportion of each group at the three stages of development is shown in Fig. 7B. There is no significant change in synaptic strength between neonates, juveniles, and adults (χ² test). Group 2 responses that occurred without an EPSP, although they appear similar, differ from antidromic APs in several ways. When the stimulus was reduced, a subthreshold EPSP was revealed in the orthodromic APs but not the antidromic APs. The antidromic responses recorded could faithfully follow frequencies of ≥50 Hz, whereas orthodromic could not.

**Trains of stimuli**

Examples of *Eₘ* recordings in response to trains of stimuli delivered to the nerve trunk, at 5, 10, 20, and 50 Hz, are presented for a neonatal, juvenile, and adult ICG neuron in Fig. 8A. The ability to faithfully follow frequencies from indirect

**FIG. 2.** A: examples of somatic action potentials (APs) recorded from a neonatal (i) and adult intracardiac neurons, one with a short afterhyperpolarization (AHPₕ₀, iii), the other with a long AHPₕ₀ (iii), evoked with brief +0.3-nA, 3-ms current pulses. Top time calibration bar refers to Ai(i) and (ii); bottom calibration bar refers to Ai(iii). B: scatterplots showing the distribution of AHPₕ₀ (i) and AHPₕ₀ (ii) recovery values of neonatal, juvenile, and adult intrinsic cardiac ganglion (ICG) neurons. C: histograms showing the AHPₕ₀ (i) and AHPₕ₀ (ii) durations for adult ICG neurons.
nerve stimuli increased with postnatal development, with 11/17 (65%) adult and 6/17 (35%) juvenile neurons being able to follow frequencies of up to 50 Hz. Synaptically evoked responses recorded from neurons in neonatal ICG exhibit a wide range of behavior. One neuron was unable to follow 0.2 Hz, another capable of faithfully following frequencies of up to 0.5 Hz (data not shown), whereas 3/8 could follow 20 Hz and only 1/6 was able to follow 50 Hz. Figure 8B graphically depicts the average number of successful APs during a 20 AP train for both indirect synaptic responses and direct somatic-evoked discharge at each stage of development.

**DISCUSSION**

This is the first study to directly examine the electrophysiological properties and synaptic transmission during postnatal maturation of a mammalian intracardiac ganglion. This investigation has made two novel findings. First we have demonstrated that there are clear changes in the passive and active properties of the ICG neurons between neonates, juveniles, and adults. Second, we have characterized synaptic transmission at the three stages of development and established that there is no significant change in synaptic transmission with postnatal development.

These experiments were performed at a temperature, divalent ion concentration, and pH buffering system relevant to in vivo conditions. By contrast, the only study to investigate these properties for the neonatal ICG (Seabrook et al. 1990) was conducted at room temperature and with high divalent ion concentrations. Therefore direct comparisons with studies of adult ICG neurons (e.g., Selyanko 1992) cannot be made. Data from dissociated neonatal and adult ICG neurons indicate that many passive and active membrane properties are temperature dependent, such as input resistance and patterns of evoked firing frequency (Cuevas et al. 1997).

The first aim of this study was to document changes in passive and active membrane properties with postnatal development. Input resistance and time constant altered with age, as did neuron size, gauged by cell capacitance with the juvenile window being approximately midway on the growth curve. The specific membrane resistance increased, in good agreement with a previous report for dissociated neonatal and adult rat ICG neurons (Hogg et al. 2001).

The decrease in specific membrane resistance accompanying postnatal development may be attributed to changes in the expression of $I_h$ and $I_M$. $I_h$ density in dissociated adult ICG neurons is approximately 50% of its value in neonatal neurons (Hogg et al. 2001). In the whole-mount preparation, application of the wide-spectrum $K^+$ channel blocker Ba$^{2+}$ (1 mM), which blocks the M-current, results in a greater decrease in slope conductance in neonatal compared with adult ICG neurons (Rimmer and Harper 2004).

Active membrane properties of neonates and adults were likewise distinct. ICG neurons in neonates possess APs with small overshoots and shallow and short-duration AHPs. In contrast, adult neurons have large overshoots and AHP amplitudes and many neurons have AHP$_{80}$ values $>50$ ms, which is not observed in neonates. Because neonates have short AHPs, they can faithfully follow higher frequencies evoked by trains of direct, intrasomatic, depolarizing currents than juvenile and adult ICG neurons.

The mean juvenile AHP duration values were midway between those for neonates and adults. This behavior is distinct from the AP overshoot and AHP amplitude, which were all marginally less than adult values. Therefore membrane properties of juvenile ICG neurons are distinct from neonates but...
FIG. 4. Voltage traces in response to a 200 ms, +0.2 nA, twice-threshold, depolarizing current pulses from neonate (A), juvenile (B), and adult neurons (C). A–C (i) illustrates examples of discharge activity classified as phasic; A–C (ii) gives examples of multiply adapting firing; and A–C (iii) show discharge activity classified as tonic. D: frequency histograms depicting the number of APs fired in response to a 200 ms twice-threshold depolarizing current pulse for neonates, juveniles, and adults.

FIG. 5. Representative voltage traces, in response to long depolarizing (+0.2 nA) and hyperpolarizing (−0.1 to −0.4 nA) 200-ms current pulses and the corresponding current–voltage plots from neonatal [A(i–iii)], juvenile [B(i–iii)], and adult [C(i–iii)] intracardiac neurons. Range of evoked discharge and time-dependent inward rectification are shown. (○) denotes steady-state measurement and (●) denotes peak hyperpolarizing from the voltage excursion measurements.
akin to adults. This indicates that evolution of the diversity of the electrophysiological properties in adult ICG neurons is not simply linked to growth of the neurons. Furthermore, the properties of juvenile ICG neurons do not change significantly during the P14–P21 time frame, inclining us to the view that the development of functional sympathetic innervation is not responsible for these changes.

The identity of the mechanism(s) underpinning the differentiation of neuron types is clearly an important area for future investigation. Developmental regulation of Ca\(^{2+}\)-/H\(^{+}\)-activated K\(^{+}\) channels (KCa) could underlie the age-dependent changes in AHP properties. The signaling pathways regulating developmental changes in the properties and expression of KCa channels in embryonic chick parasympathetic ganglion neurons has been the subject of a series of elegant studies (Cameron and Dryer 2000; Cameron et al. 1998, 1999; Dryer 1998). A similar analysis is required for mammalian ICG neurons.

We can infer that an increase in SKCa channel expression underpins the changes in AHP duration that accompany development, from the results of experiments using the SK channel blocker apamin. Apamin reduced both adult and neonatal AHP\(_{50}\) and AHP\(_{80}\) durations to almost the same value. Apamin also switched the discharge characteristics from phasic to tonic in neonates and adults, implicating a role for SK channels in control of firing. In contrast, apamin produced no change in firing discharge in dissociated neonatal rat intracardiac neurons (Cuevas et al. 1997). An increase in SKCa current may also contribute to the small change in distribution of evoked firing from neonates to adults (Fig. 4B). Discharge activity is primarily controlled by the activity of the M-channel in dissociated neonatal neurons (Cuevas et al. 1997). Differential densities of the M-current may also contribute to this behavior.

The presence of TDR evoked by hyperpolarizing current pulses is held as the signature of I\(_{h}\) (Pape 1996). There was an increase in TDR with postnatal development, which is apparently inconsistent with the results of Hogg et al. (2001). Other voltage-sensitive currents operating in this voltage range could be contributing to the membrane potential trajectory. Clearly, voltage-clamp studies in the intact ganglion are required to resolve this apparent discrepancy.

Spontaneous synaptic and action potential discharge was recorded from a population of neurons, as reported previously (Edwards et al. 1995; Xi-Moy et al. 1993). There are no reports of spontaneous EPSPs, action potential discharge, or pacemaker firing in cultured or dissociated ICG neurons (Adams and Cuevas 2004). The spontaneous activity recorded in this study was blocked by application of 100 \(\mu\)M Cd\(^{2+}\), indicating such activity is synaptically evoked rather than originating from damage arising from the impalement.
The second component of the study charted synaptic transmission from the neonate to the adult ganglion. Several types of postsynaptic responses were recorded from ICG neurons in response to a single presynaptic stimulus. These were divided into three classes: weak, secure, and strong based on the ability to fire an AP and the presence and timing of the secondary EPSP in relation to the action potential. A similar scheme was adopted for rat submandibular neurons (Smith et al. 2000). Proportions of these synaptic responses recorded from ICG neurons at different stages of postnatal development were not significantly different. Maximum action potential following frequency was higher for indirect, synaptically evoked events than for those generated by brief intrasomatic depolarizing current pulses, presumably because of increased conductance resulting from the underlying EPSP. The test frequency range (5–50 Hz) examined in the present study was used in previous investigations to stimulate the cardiac vagus in the rat (Jones et al. 1998) and was recorded during efferent reflex discharge in the cat (Kunze 1972), and thus can be regarded as being physiologically relevant. The ability of synaptically evoked action potentials to follow high-frequency repetitive stimulation shows modest improvement with postnatal development.

Functional significance

This work has sought to increase knowledge regarding somatic membrane properties and synaptic transmission within mammalian ICG. This has several implications, among which is how changes within the neural circuit during postnatal development affect heart function. It is becoming clear that local neural circuits may be important in setting up arrhythmic substrates. There are numerous clinical case reports of cardiac brady-arrhythmias arising from activity in the CNS and traversing the parasympathetic ganglia, such as during or after epileptic seizures (Rugg-Gunn et al. 2004). ICGs need to be considered as part of the pathway that sets up conditions for such arrhythmias. Therefore further analysis of the circuitry and synaptic transmission within intracardiac neurons and interactions with sympathetic and central nervous systems should promote further understanding regarding control of the heart during development and disease. For example, several studies have reported abnormalities in ganglionic transmission in the chronic rapid ventricular pacing model of heart failure (Bibevski and Dunlap 1999, 2004).

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REFERENCES


