Developmental Changes in Hyperpolarization-Activated Currents $I_h$ and $I_{K(IR)}$ in Isolated Rat Intracardiac Neurons

R. C. HOGG, A. A. HARPER, AND D. J. ADAMS

School of Biomedical Sciences, Department of Physiology and Pharmacology, University of Queensland, Brisbane, Queensland 4072, Australia

Received 22 December 2000; accepted in final form 14 March 2001

Hogg, R. C., A. A. Harper, and D. J. Adams. Developmental changes in hyperpolarization-activated currents $I_h$ and $I_{K(IR)}$ in isolated rat intracardiac neurons. J Neurophysiol 86: 312–320, 2001. The hyperpolarization-activated nonselective cation current, $I_h$, was investigated in neonatal and adult rat intracardiac neurons. $I_h$ was observed in all neurons studied and displayed slow time-dependent rectification. $I_h$ was isolated by blockade with external Cs$^+$ in all neurons studied and displayed slow time-dependent rectification. $I_h$ was approximately twofold greater in neurons from adult (−2.3 pA/pF per well) as compared with adult (−2.3 pA/pF) rats; however, the reversal potential and activation parameters were unchanged. The reversal potential and amplitude of $I_h$ was sensitive to changes in external Na$^+$ and K$^+$ concentrations. An inwardly rectifying K$^+$ current, $I_{K(IR)}$, was also present in intracardiac neurons from adult but not neonatal rats and was blocked by extracellular Ba$^{2+}$. $I_{K(IR)}$ was present in approximately one-third of the adult intracardiac neurons studied, with a current density of −0.6 pA/pF at −130 mV. $I_{K(IR)}$ displayed rapid activation kinetics and no time-dependent rectification consistent with the rapidly activating, inward K$^+$ rectifier described in other mammalian autonomic neurons. $I_{K(IR)}$ was sensitive to changes in external K$^+$, whereby raising the external K$^+$ concentration from 3 to 15 mM shifted the reversal potential by approximately +36 mV. Substitution of external Na$^+$ had no effect on the reversal potential or amplitude of $I_{K(IR)}$. $I_{K(IR)}$ density increases as a function of postnatal development in a population of rat intracardiac neurons, which together with a concomitant decrease in $I_h$ may contribute to changes in the modulation of neuronal excitability in adult versus neonatal rat intracardiac ganglia.

INTRODUCTION

Autonomic control of heart rate changes during early postnatal development in the rat. Direct vagal nerve stimulation studies reveal significant decreases with postnatal age in the intrinsic heart rate and maximal parasympathetic control of heart rate (Quigley et al. 1996). The electrical properties of autonomic ganglion neurons from immature animals have been reported to be different from those of mature, adult animals (e.g., Gottmann et al. 1988; Hirst and McLachlan 1984). The intrinsic membrane properties of a neuron are not static but may alter during development by the up or downregulation of ion channel expression or as a result of modulation of the existing complement of ion channel subunits. Membrane hyperpolarization can activate two inwardly rectifying conductances in autonomic neurons: a nonselective cation current ($I_h$), with Na$^+$ and K$^+$ as the charge carriers, and a K$^+$-selective current ($I_{K(IR)}$).

Electrophysiological studies have revealed that $I_h$ is expressed in all neonatal (Cuevas et al. 1997) and adult (Xi-Moy and Dunn 1995) rat intracardiac neurons. $I_h$ was originally identified in mouse primary afferent neurons (Mayer and Westbrook 1983), and is analogous to $I_f$ in the cardiac conduction system (DiFrancesco et al. 1986), which contributes to the generation of the cardiac rhythm. In guinea pig intracardiac ganglia, the population of neurons possessing $I_h$ frequently display an inherent repetitive spontaneous discharge and have been proposed to serve a sensory role (Edwards et al. 1995).

Inwardly rectifying K$^+$ channels open on membrane hyperpolarization and close with membrane depolarization. The inwardly rectifying K$^+$ current, first characterized in rabbit sympathetic ganglion neurons as a time-independent rectification of hyperpolarizing electrotonic potentials (Christ and Nishi 1973), has been identified in a variety of mammalian autonomic neurons (see Adams and Harper 1995). Whereas both $I_h$ and $I_{K(IR)}$ are activated by hyperpolarization and are sensitive to the extracellular K$^+$ concentration, they exhibit different voltage dependence, kinetics, and pharmacological sensitivity. $I_{K(IR)}$ activation is voltage dependent with a threshold of approximately −85 mV in physiological extracellular K$^+$ concentrations and effectively instantaneous kinetics. In contrast, $I_h$ activates slowly, typically with a time constant of tens of milliseconds at voltages negative to −60 mV, and the time course of activation is voltage sensitive. $I_h$ is reversibly blocked by external Cs$^+$ (≈1 mM) but not by Ba$^{2+}$, distinguishing it from $I_{K(IR)}$, which is blocked by low micromolar Ba$^{2+}$ concentrations (Adams and Nonner 1990). The reversal potential of $I_h$ is positive to the resting membrane potential ($E_m$), ranging between −50 and −20 mV (Pape 1996), whereas the reversal potential of $I_{K(IR)}$ follows the K$^+$ equilibrium potential ($E_K$). These characteristics suggest that $I_h$ and $I_{K(IR)}$ may be suited to modulate the subthreshold resting potential.

The expression of $I_h$ and $I_{K(IR)}$ has been reported to vary significantly in adult rat dorsal root ganglion (DRG) neurons of different size (Petruska et al. 2000; Scroggs et al. 1994), and regional differences in the distribution of the hyperpolarization-activated currents, $I_h$ and $I_{K(IR)}$, have been demonstrated in the...
rat primary afferent neurons (Wang et al. 1997). $I_h$ was largely confined to the soma, whereas $I_{K(IR)}$ was less frequently found in the soma than in the growth cone. This inhomogeneous distribution of these ion channels is consistent with the differential functions of these currents. The aim of this present study was to determine whether the expression and properties of $I_h$ and $I_{K(IR)}$ are related to the stage of postnatal development in rat intracardiac neurons and subsequently to determine whether these currents are involved in control of firing behavior. Preliminary reports of some of these results have been presented to the Physiological Society (Harper et al. 1998; Hogg et al. 1999).

METHODS
Preparation

The isolation and culture of parasympathetic neurons from neonatal rat intracardiac ganglia has been described previously (Xu and Adams 1992). Briefly, neonatal rats (2–8 days old) were killed by decapitation in accordance with the guidelines of the University of Queensland Animal Experimentation Ethics Committee. The heart was excised and placed in a saline solution containing (in mM) 140 NaCl, 3 KCl, 2.5 CaCl$_2$, 0.6 MgCl$_2$, 7.7 glucose, and 10 histidine (pH 7.2). Atria were separated and the medial region containing the pulmonary veins and superior vena cava was identified, isolated, and incubated in a saline solution containing collagenase (1 mg/ml, Worthington–Biochemical Type 2, specific activity ~100 units/mg) at 37°C for 60 min. Cross-sections of ganglia were dissected from the epicardial ganglion plexus and dispersed by trituration in a high glucose culture medium (Dulbecco’s modified Eagle medium), containing 10% fetal calf serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin. The dissociated neurons were plated on to laminin-coated glass coverslips and incubated at 37°C under a 95% air-5% CO$_2$ atmosphere for 24–60 h. In a series of experiments, intracardiac neurons were isolated from neonatal rats using trypsin (0.2 mg/ml) in the dissociation procedure and the same enzymatic conditions used for obtaining adult neurons.

Young adult female Wistar rats (5–6 wk, 160–180 g) were killed by stunning and cervical dislocation, the hearts excised, atria isolated and placed in cold saline solution. The intracardiac ganglia were identified and dissected from the fat pads of the dorsal surfaces of the atria. Intracardiac neurons were isolated using a combination of enzymatic and mechanical dispersion using a protocol adapted from that described previously (Jeong and Wurster 1997). The ganglia were incubated in a saline solution containing 1.2 mg/ml collagenase (specific activity ~1,000 units/mg; Sigma Type 1A) and 0.1–0.2 mg/ml trypsin from bovine pancreas (~13,000 units/mg; Sigma) at 37°C for 60 min. The ganglia were dispersed by trituration and washed twice with culture medium. The protocol for plating and incubation followed that described for neonatal neurons.

Electrophysiological recording

Neurons plated on glass coverslips were transferred to a recording chamber (volume 0.5 ml) mounted on an inverted phase contrast microscope (x400 magnification), allowing isolated neurons to be identified. Membrane voltage and current were recorded using the perforated patch whole cell recording configuration. The perforated patch recording configuration was used to preserve intracellular regulatory systems and reduce “rundown” or loss of membrane currents through cell dialysis. Our previous studies have demonstrated that some ionic currents including $I_h$ are absent in dialyzed intracardiac neurons (Cuevas et al. 1997). A final concentration of 240 μg/ml amphotericin B in 0.4% DMSO was used in the pipette solution. Patch electrodes were pulled from thin-walled borosilicate glass (GC150TF; Harvard Apparatus Ltd., Edenbridge, Kent, UK) and after fire-polishing had resistances of ~1 MΩ. Access resistances using the perforated patch configuration were routinely ≤2 MΩ following series resistance compensation. Membrane current and voltage were recorded using an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA) and were filtered at 10 and 3 kHz, respectively, then digitized at 10–50 kHz (Digidata 1200A interface, Axon Instruments) and stored on the hard disk of a PC for viewing and analysis. Voltage and current protocols were applied using pClamp software (Version 6.1.3, Axon Instruments). Boltzmann fits were made using a least-squares nonlinear curve fitting routine in Microcal Origin 5.0 (Microcal Software, Northampton, MA), and straight line fits were by linear regression. No corrections were made for liquid junction potentials. Data are presented as means ± SE and are compared using paired or unpaired t-tests.

Solutions

The control external solution for perforated-patch whole cell recordings was physiological saline solution (PSS) containing (in mM) 140 NaCl, 3 KCl, 2.5 CaCl$_2$, 1.2 MgCl$_2$, 7.7 glucose, and 10 HEPES–NaOH buffered to pH 7.2. The pipette solution contained (in mM), 75 K$_2$SO$_4$, 55 KCl, 5 MgSO$_4$, and 10 HEPES (titrated to pH 7.2 with N-methyl-D-glucamine). Alterations in extracellular K$^+$ concentration were made by equimolar substitution of K$^+$ for Na$^+$. Changes in extracellular Na$^+$ were made by replacing extracellular Na$^+$ with either N-methyl-D-glucamine (NMDG) or arginine. The osmolarity of the extracellular and pipette solutions was monitored by a vapor pressure osmometer (Wescor 5500, Logan, UT) and were in the range 280–290 mmol/kg. The temperature of the superfusing solutions was controlled by a Peltier thermoelectric device and monitored by an independent thermistor probe in the recording chamber. The recording chamber was continuously superfused with a maximum deviation of 1°C in any individual procedure. Pharmacological agents were bath applied at the concentrations indicated. All chemicals used were of analytical grade. The following drugs were used: amphotericin B (Sigma Chemical, St. Louis, MO), N-ethyl-1,6-dihydro-1,2-dimethyl-6-(methylamino)-N-phenyl-4-pyrimidinamine hydrochloride (ZD 7288; Tocris Cookson, Bristol, UK).

RESULTS

Passive electrical properties of intracardiac neurons from neonatal and adult rats

Hyperpolarization-activated currents were studied in a total of 25 neonatal and 16 adult rat intracardiac neurons. Neurons isolated from neonatal rats were on average smaller in size than those from adults having a mean cell capacitance of 19.0 ± 3.4 pF (n = 12) compared with 54.3 ± 8.5 pF (n = 8) for adult neurons. The mean cell input resistance was higher in neonatal than adult neurons, and there was no significant difference in the resting membrane potential (see Table 1).

Current-clamp recording of $I_h$ and $I_{K(IR)}$

$I_h$ was evident in current-clamp records as a characteristic sag in the voltage response to a hyperpolarizing current step (Fig. 1A). Peak and steady-state values for the voltage response in an adult rat intracardiac neuron are plotted in Fig. 1D for control (● and ○) and in the presence of 10 μM Ba$^{2+}$ (▼ and ▼) to inhibit $I_{K(IR)}$. External Ba$^{2+}$ (10 μM) slightly increased the membrane resistance and caused a leftward shift in the current-voltage (I–V) relationship of a similar magnitude for
TABLE 1. Passive electrical properties of intracardiac neurons from neonatal and adult rats

<table>
<thead>
<tr>
<th></th>
<th>Neonate</th>
<th>Adult</th>
<th>P Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell capacitance, pF</td>
<td>19.0 ± 3.4 (12)</td>
<td>54.3 ± 8.5 (8)</td>
<td>0.0003</td>
</tr>
<tr>
<td>E&lt;sub&gt;rev&lt;/sub&gt;, mV</td>
<td>-52.5 ± 1.8 (11)</td>
<td>-52.4 ± 1.3 (10)</td>
<td>ns</td>
</tr>
<tr>
<td>R&lt;sub&gt;m&lt;/sub&gt;, MQ</td>
<td>534 ± 75 (9)</td>
<td>321 ± 47 (6)</td>
<td>ns</td>
</tr>
<tr>
<td>R&lt;sub&gt;m&lt;/sub&gt;, kΩ·cm&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>8.3 ± 1.62 (7)</td>
<td>15.3 ± 2.8 (4)</td>
<td>0.045</td>
</tr>
</tbody>
</table>

Values are means ± SE; number of neurons are in parentheses. Unpaired t-test was used for P values. ns, not significant. R<sub>m</sub> values calculated for −50 pA hyperpolarizing current steps. R<sub>m</sub> values calculated from R<sub>m</sub> × cell capacitance, assuming 1 pF = 10 μm<sup>2</sup>. Temperature, 22°C.

Both peak and steady-state voltage responses to hyperpolarizing currents (Fig. 1D).

External Cs<sup>+</sup> (2 mM) reversibly inhibited the sag in the voltage response (Fig. 1C). The effect of 2 mM Cs<sup>+</sup> on the steady-state I-V relationship is shown in Fig. 1E. External Cs<sup>+</sup> caused an increase in the membrane resistance in the activation range of I<sub>h</sub>. The block of I<sub>h</sub> with 2 mM Cs<sup>+</sup> produced a small, but significant, hyperpolarization of membrane potential at both 22°C (−52.9 ± 2.0 mV control, mean ± SE; −54.7 ± 1.8 mV, Cs<sup>+</sup>, n = 6, P = 0.04) and 37°C (−50.0 ± 2.9 mV control; −52.6 ± 3.1 mV, Cs<sup>+</sup>, n = 5, P = 0.01). Under the same conditions, Cs<sup>+</sup> lengthened the duration between action potentials within bursts evoked by a sustained depolarizing current pulse and lengthened the afterhyperpolarization (AHP) following single action potentials evoked by a short depolarizing pulse (500 pA, 2.5 ms). The frequency of action potential discharge in response to depolarizing currents was slowed by 12% in the presence of external Cs<sup>+</sup> at 37°C; the interval between the first and second action potential being 20.1 ± 1.7 ms (control) and 22.6 ± 1.7 ms (Cs<sup>+</sup>; n = 4) but was not affected at 22°C (Fig. 2). Similarly, whereas the time for recovery of the action potential AHP (AHP duration to 80% recovery) at 37°C significantly increased from 22.1 ± 5.9 ms (control) to 28.2 ± 6.6 ms (n = 3; P = 0.03) in the presence of Cs<sup>+</sup>, no change in AHP duration was observed at 22°C.

Temperature-dependent effects of Cs<sup>+</sup> on membrane potential, spike interval, and the AHP were similar in intracardiac neurons isolated from adult and neonatal rats.

Pharmacological isolation of I<sub>h</sub>

I<sub>h</sub> was isolated under voltage-clamp conditions in neurons from neonatal and adult rats as a slowly activating inward current in response to hyperpolarizing voltage steps negative to −60 mV. I<sub>h</sub> was reversibly inhibited by the addition of 2 mM CsCl to the bathing solution and could be isolated by subtraction of currents recorded in the presence of Cs<sup>+</sup> from those obtained in control conditions (Fig. 3A). The bradycardic agent ZD 7288 (10–100 μM), which has been shown to block I<sub>h</sub> and I<sub>h</sub> (BoSmith et al. 1993; Harris and Constanti 1995) also inhibited I<sub>h</sub> in rat intracardiac neurons in a concentration-dependent and irreversible manner (Fig. 3B).

Several distinct protocols for determining the I-V relationship of I<sub>h</sub> have been described, and the measurement of the reversal potential with the protocol used (Wang et al. 1997) was in agreement with that calculated using the other methods (Jafri and Weinrich 1998; Lamas 1998). Figure 3C shows an I-V relationship for I<sub>h</sub> in a neonatal neuron determined by subtraction of the total current I-V relationship in control conditions from that in the presence of 2 mM Cs<sup>+</sup>. Figure 3D shows the difference I-V relationship in an adult neuron obtained in the absence and presence of 100 μM ZD 7288. There was no significant difference in the reversal potential of I<sub>h</sub> obtained in the presence of either Cs<sup>+</sup> or ZD 7288 (see Table 2).

The current density of I<sub>h</sub> was twofold greater in neonatal than in adult rat intracardiac neurons (Fig. 4, A and B; see Table 2). The relationship between steady-state current and cell size (pF) was well described by a linear regression for each stage of development (R = 0.93 and 0.89 for neonatal and adult neurons, respectively). Representative traces of I<sub>h</sub> obtained from neonatal (23 pF) and adult (63 pF) neurons are shown in Fig. 4, A and B.

I<sub>h</sub> has been reported to be absent in neurons dissociated from neonatal rat cerebral cortex immediately following cell isolation.

FIG. 1. Inward and time-dependent rectification in current-clamped intracardiac neurons from adult rats. A–C: Voltage responses obtained in response to depolarizing and hyperpolarizing current pulses pulses (+100, −50, −100, and −150 pA for A and B, and +100, −50, and −100 pA for C) are shown for control conditions, in the presence of 10 μM Ba<sup>2+</sup> and 2 mM Cs<sup>+</sup>. Temperature, 22°C. D and E: Current-voltage (I-V) relations for each maneuver plotted for the peak voltage response (∙ and ○) and the steady-state response measured at the end of the current pulse (∗ and ▲).
and reappears after 1–2 days in culture (Budde et al. 1994). The disappearance of $I_h$ was attributed to the use of trypsin in the dissociation procedure, and $I_h$ was also sensitive to proteolysis following superfusion of the preparation with trypsin. In a series of experiments, collagenase and trypsin were used to dissociate neonatal intracardiac neurons. $I_h$ was present in all neonatal neurons dissociated with trypsin and collagenase with a current density of $25.6 \pm 1.2 \text{ pA/pF (n=6)}$, which was not significantly different ($P > 0.05$) from the mean value obtained for neonatal neurons dissociated with collagenase only (cf. Table 2).

| TABLE 2. Characteristics of $I_h$ and $I_{K(IR)}$ in neonatal and adult rat intracardiac neurons |
|----------------|----------------|----------------|
| $I_h$, pA pF$^{-1}$ | $-4.1 \pm 0.3 (12)$ | $-2.3 \pm 0.3 (8)$ |
| $I_h$, $E_{rev}$, mV | $-22.2 \pm 7.0 (3)$ | $-23.6 \pm 3.6 (7)$ |
| $G_{max, K}$, pS pF$^{-1}$ | $42.3 \pm 8.4 (3)$ | $22.2 \pm 3.0 (7)$ |
| $E_{rev}$, mV | $-84.0 \pm 3.0 (4)$ | $-74.8 \pm 3.7 (4)$ |
| $k$, mV | $11.2 \pm 0.6 (4)$ | $12.8 \pm 1.5 (4)$ |
| $P_{Na}/P_K$ | $0.11 (4)^*$ | $0.10 (2)$ |
| $I_{K(IR)}$, pA pF$^{-1}$ | $0.61 \pm 0.26 (6)$ |

Values are means ± SE; number of neurons are in parentheses. $I_h$ and $I_{K(IR)}$ current density determined at $-130 \text{ mV}$. Temperature, $22^\circ \text{C}$. $E_{rev}$, reversal potential; $G_{max, K}$, fully activated conductance density; $E_{K}$ and $k$ indicate the midpoint and inverse slope factor at the midpoint of the activation curve. Data were compared using $t$-tests; ns, not significant. * Data obtained from Cuevas et al. (1997).

and reappears after 1–2 days in culture (Budde et al. 1994). The disappearance of $I_h$ was attributed to the use of trypsin in the dissociation procedure, and $I_h$ was also sensitive to proteolysis following superfusion of the preparation with trypsin. In a series of experiments, collagenase and trypsin were used to dissociate neonatal intracardiac neurons. $I_h$ was present in all neonatal neurons dissociated with trypsin and collagenase with a current density of $-5.6 \pm 1.2 \text{ pA/pF (n=6)}$, which was not significantly different ($P < 0.05$) from the mean value obtained for neonatal neurons dissociated with collagenase only (cf. Table 2).

![FIG. 2. Effect of temperature on somatic interaction potential interval. A and B: superimposed traces of action potential discharges evoked by depolarizing current pulses ($+100 \text{ pA, } 1 \text{ s}$) obtained at 22 and 37°C in control solution and in the presence of 2 mM Cs$^+$.](http://jn.physiology.org/)

![FIG. 3. Isolation of $I_h$ in voltage-clamped neonatal and adult rat intracardiac neurons. Currents were evoked by 2-s hyperpolarizing steps to test potentials between $-60$ and $-130 \text{ mV}$ from a holding potential of $-50 \text{ mV}$. Temperature, $22^\circ \text{C}$. A: $I_h$ recorded from a neonatal neuron in control conditions and after addition of 2 mM CsCl to the extracellular solution. The Cs$^+$-sensitive current was obtained by subtracting the currents recorded in the presence of Cs$^+$ from control. B: $I_h$ recorded from an adult neuron in control conditions and in the presence of $100 \mu \text{M ZD 7288}$. The ZD 7288-sensitive current was obtained by subtraction. C and D: $I-V$ relations of steady-state currents recorded in control and 2 mM Cs$^+$ or $100 \mu \text{M ZD 7288}$ and the Cs$^+$- or ZD 7288-sensitive component.](http://jn.physiology.org/)
Characterization of $I_{K(IR)}$ in adult neurons

A rapidly activating inwardly rectifying current, $I_{K(IR)}$, was present in approximately one-third (6 of 16 cells) of the adult rat intracardiac neurons studied but not in neurons obtained from neonatal rats ($n = 25$). $I_{K(IR)}$ was blocked by bath application of solutions containing 10 μM Ba$^{2+}$. $I_{K(IR)}$ displayed rapid activation kinetics but did not exhibit time-dependent rectification consistent with the rapidly activating inward K$^+$ rectifier described in other mammalian autonomic neurons (see Adams and Harper 1995). Under current-clamp conditions, 10 μM Ba$^{2+}$ reduced both the peak and steady-state voltage response to hyperpolarizing current pulses to a similar degree (Fig. 1). The Ba$^{2+}$-sensitive component was isolated by subtraction of currents obtained in the presence of 10 μM Ba$^{2+}$ from the total current. Figure 5A shows that the Ba$^{2+}$-sensitive current, $I_{K(IR)}$, was not affected by the $I_h$ inhibitor, ZD 7288 (100 μM). $I_{K(IR)}$ was sensitive to changes in external K$^+$ but not Na$^+$. Raising external K$^+$ from 3 to 15 mM shifted the reversal potential by approximately +36 mV (Fig. 5C) similar to that predicted by the Nernst equation for a K$^+$-selective electrode. $I_{K(IR)}$ had a current density of $-0.61 \pm 0.26$ pA/pF ($n = 6$) at $-130$ mV in 3 mM extracellular K$^+$. Activiation characteristics of $I_h$

The steady-state $I-V$ relationship for $I_h$ was determined from tail current amplitude following maximal current activation and was linear in both neonatal and adult neurons (Fig. 6, A and B). The reversal (zero-current) potential was determined by extrapolation of the $I-V$ relationship for tail currents. There was no significant difference in the reversal potentials obtained for $I_h$ in neonatal ($-22.2 \pm 7.0$ mV, $n = 3$) and adult ($-23.6 \pm 3.6$ mV, $n = 7$) rat intracardiac neurons, respectively. The mean slope conductance was $42.3 \pm 8.4$ pS/pF ($n = 3$) for neonates and $22.2 \pm 3.0$ pS/pF ($n = 7$) for adults.

The steady-state activation characteristics of $I_h$ were compared in voltage-clamped neonatal and adult intracardiac neurons. Neurons were stepped from test potentials between $-60$ and $-120$ mV to $-50$ mV (Fig. 6C). Tail current amplitudes were normalized to the maximum current measured at $-120$ mV. The relationship between the prepulse potential and the normalized current amplitude was fit by a Boltzmann equation (Fig. 6D). There was no significant difference in the slope or midpoint of the activation curves of $I_h$ from neonatal or adult neurons (see Table 2).

The activation kinetics of $I_h$ were compared in neonatal and adult neurons, and the activation time course was best fit by a single exponential. The time course of activation was voltage dependent, becoming more rapid with increasing membrane...
hyperpolarization (Fig. 7A). The time constant of activation \( (\tau) \) for \( I_h \) between \(-130 \) and \(-100 \) mV is plotted against membrane potential as shown in Fig. 7B. \( \tau \) was significantly faster in intracardiac neurons from adult (89 ± 16 ms, \( n = 11 \)) as compared with neonatal rats (263 ± 32 ms, \( n = 8 \)) at \(-130 \) mV and 22°C and exhibited an e-fold change per 27.0 mV in adult and per 28.5 mV in neonatal rat neurons.

**Effect of temperature on \( I_h \)**

The properties of \( I_h \) in neurons from adult and neonatal rats were examined at 22 and 37°C under voltage-clamp conditions. The time constant of activation decreased exponentially with increasing hyperpolarization (Fig. 8A) and exhibited an e-fold change per 24 mV at 22°C and 18 mV at 37°C. Raising the temperature from 22 to 37°C significantly reduced the time constant of activation at \(-130 \) mV in neurons from neonatal rats (Fig. 8B) from 263 ± 32 ms (\( n = 3 \)) to 46 ± 7 ms (\( n = 3 \)) giving a \( Q_{10} \) of 3.1. The current density of \( I_h \) measured at 22°C was significantly greater in neurons from neonatal (\(-4.1 \) pA/pF at \(-130 \) mV) as compared with adult (\(-2.3 \) pA/pF) rats. The current density of \( I_h \) in neurons from neonatal rats increased to \(-6.3 \) pA/pF at 37°C and exhibited a \( Q_{10} \) of 1.3.
Ionic basis of \( I_h \)

\( I_h \) was sensitive to changes in the extracellular K\(^+\) concentration, whereby elevating external K\(^+\) from 3 to 15 mM caused an approximate threefold increase in the amplitude of the inward current in response to hyperpolarizing voltage steps. Raising external K\(^+\) had no effect on the time course of activation (Fig. 9A). The fully activated and steady-state \( I-V \) relationships were determined for adult neurons in 3 and 15 mM extracellular K\(^+\) (Fig. 9, B and C). A fivefold increase in the extracellular K\(^+\) concentration caused a four- to fivefold increase in the slope conductance without a significant shift of the reversal potential (Fig. 9B). The calculated \( P_{Na}/P_K \) ratio obtained in the presence of 3 mM K\(^+\) and 15 mM K\(^-\) was 0.42 and 0.35, respectively. Replacement of extracellular Na\(^+\) with either NMDG or arginine shifted the \( I-V \) relationship and reversal potential to more negative membrane potentials, indicating that Na\(^+\) contributes to \( I_h \) in these neurons (\( n = 5 \), data not shown). Elevating the extracellular K\(^+\) concentration did not affect the steady-state activation characteristics of \( I_h \). Steady-state activation curves obtained from adult rat intracardiac neurons are shown in Fig. 9D.

**DISCUSSION**

We have analyzed two distinct hyperpolarization-activated currents in rat intracardiac neurons, \( I_h \) and \( I_{K(IR)} \). \( I_h \) was identified on the basis of its pharmacological and biophysical properties that are similar to a nonselective cation current \( I_h \) described in central and peripheral neurons (see Pape 1996) and was present in intracardiac neurons from neonatal and adult rats. Another inwardly rectifying K\(^+\)-selective current, \( I_{K(IR)} \), was observed in intracardiac neurons from adult rats only. Neonatal and adult intracardiac neurons differed in size with the mean cell capacitance of adult neurons more than twofold higher than neonatal. \( I_h \) current density was significantly lower in adult rat intracardiac neurons, which coincided with the appearance of \( I_{K(IR)} \) in some neurons from adult rats.

In previous experiments using the conventional dialyzed whole cell recording configuration, \( I_h \) was not observed, indicating that a diffusible intracellular factor may be required (Xu and Adams 1992). The lower current density of \( I_h \) in neurons from adult rats was not due to disappearance of \( I_h \) caused by the use of trypsin during dissociation (cf. Budde et al. 1994) as intracardiac neurons isolated from neonatal rats using collagenase and trypsin did not exhibit a significantly lower density of \( I_h \) than for neurons isolated using collagenase only. Changes in expression of \( I_h \) with development have been reported previously whereby an increase in the number of cells expressing \( I_h \) increases during development in embryonic quail ganglion neurons (Schlichter et al. 1991). Age-related changes in \( I_h \) have also been described in rat hypoglossal motoneurons (Bayliss et al. 1994), and in rabbit sinoatrial node cells the slope conductance of \( I_h \) is greater in newborn than in adult rabbits (Accili et al. 1997).

Although \( I_h \) has been reported previously in intracardiac neurons from both neonatal (Cuevas et al. 1997) and adult rats (Xi-Moy and Dun 1995), the reversal potential, ionic selectivity, and activation characteristics of \( I_h \) have not been described. In vitro studies of autonomic neurons are often carried out at room temperature (20–22°C); however, the temperature dependence of ionic currents is often nonlinear, and different ionic conductances can have distinct temperature sensitivities. This results in conductances having differing effects on neuronal firing at 22 and 37°C. The effect of temperature on \( I_h \) indicates that the kinetics of \( I_h \) activation is highly temperature dependent, whereas current density was, in contrast, less sensitive to temperature change. The increased AHP and interval between action potentials at 37°C but not 22°C following block of \( I_h \) with Cs\(^+\) suggest that \( I_h \) contributes to the control of neuronal excitability primarily at 37°C.

Our studies have shown that the firing behavior of adult intracardiac neurons is different from that observed in neonatal neurons. Adaptive firing observed at 37°C in intracardiac neurons from neonatal rats has been shown to be regulated by the
muscarnine-sensitive K⁺ current (Iₘ) (Cuevas et al. 1997), whereas the firing behavior of neurons from adult rats under similar conditions is not altered in the presence of ACh (Hogg and Adams, unpublished observation). Differences in current density and activation kinetics of Iₘ may contribute to this change in firing properties. Iₘ is likely to be activated during the AHP of the action potential, and, given that the reversal potential is positive to the Eₘ, it will act to depolarize the cell, limit the AHP duration, and shorten the interval between action potentials to promote multiple discharge. The effects of external Cs⁺ on the Eₘ and action potential discharge at 37°C suggest that Iₘ may play a role in regulating action potential firing in intracardiac neurons. Iₘ has been reported to contribute to the Eₘ in rat superior cervical ganglion sympathetic neurons (Lamas 1998) and contribute to the resting and active properties of sensory (Jafri and Weinrich 1998) and central neurons (Maccaferri et al. 1993; McCormick and Pape 1990; Solomon and Nerbonne 1993; Womble and Moises 1993). Iₘ is subject to modulation by neurotransmitters, neuropeptides, and inflammatory mediators that converge to act via adenylic cyclase (Pape 1996). The membrane permeable analogue of cAMP, 8-bromo cAMP, increased the amplitude of Iₘ in neonatal rat intracardiac neurons (Hogg and Adams, unpublished observations). It is possible that Iₘ plays a more significant role in resting and active properties in intracardiac neurons in vitro due to neuro-modulation than is apparent in isolated neurons.

Iₖ(IR) activates instantaneously, does not exhibit time-dependent rectification, and is blocked by externally applied Ba²⁺ (10 μM) similar to the inward rectifying K⁺ current recorded in numerous neuron types (see Adams and Harper 1995; Rudy 1988). The precise role of Iₖ(IR) in adult intracardiac neurons remains unclear. The activation range of Iₖ(IR) would confine its activation to membrane potentials negative to Eₘ, such as would occur during the action potential AHP. The differences in firing behavior in neonatal and adult rat intracardiac neurons are likely due to changes in the contribution of both Iₘ and Iₖ(IR).

The ion channel inhibitors, Cs⁺ and Ba²⁺, have been shown to differentially affect the vagally induced pacemaker response in anesthetized dogs, whereby BaCl₂ attenuated the vagally induced bradycardia without affecting other components of the response. In contrast, CsCl had no effect on the initial vagal slowing of atrial rate but abolished the acceleratory portion of the response (Wallick et al. 1997). Local arterial infusion of barium chloride to the right atrial ganglionated plexus has also been shown to directly modulate the electrical activity of canine intracardiac neurons in situ (Thompson et al. 2000). The inhibition of Iₘ and Iₖ(IR) by Cs⁺ and Ba²⁺, respectively, may
contribute to the observed changes in neuronal excitability of mammalian intrinsic cardiac ganglia. Furthermore, the changes in the functional expression of $I_h$ and $I_{KIR}$ with postnatal development suggests that different ionic mechanisms may contribute to modulating neuronal excitability neonatal and adult intracardiac ganglion neurons.

This work was supported by National Health and Medical Research Council of Australia and Australian Research Council grants to D. J. Adams and a Travel Research Award from The Royal Society and The Carnegie Trust for the Universities of Scotland to A. A. Harper.

Present address of A. A. Harper: Div. of Molecular Physiology, School of Life Sciences, University of Dundee, Dundee DD1 4HN, UK.

REFERENCES


