Age-Related Appearance of Outward Currents May Contribute to Developmental Differences in Ventricular Repolarization

Cynthia D. Jeck and Penelope A. Boyden

Ventricular repolarization significantly influences contractility, refractoriness, and ion channel state. Factors affecting repolarization will thus affect these secondary phenomena. To understand the influence of age on ventricular repolarization, we studied neonatal, young, and adult dogs using electrocardiogram, action potential, and whole-cell voltage-clamp recordings from single epicardial myocytes. Hearts of neonatal and 57–58 day-old dogs require a significantly longer time for repolarization than those of adult dogs, as determined by analysis of rate-corrected QT and JT (QT minus QRS) intervals. Epicardial action potentials of neonates are significantly longer than those of adults, as determined by measurements of duration at 50% and 90% repolarization. The adult action potential is characterized by a large phase 1 notch that is absent from neonatal recordings. This notch develops between 58 and 64 days of age, and by 64–68 days of age, it is equal to that in adults. In addition, action potentials recorded from adult and young epicardial muscle are more greatly affected by rapid pacing and superfusion of 2 mM 4-aminopyridine than are potentials recorded from neonatal tissue. Whole-cell voltage-clamp recordings reveal a 4-aminopyridine–sensitive transient outward current in adult myocytes that is absent from neonatal myocytes. The correlation between developmental changes in the 4-aminopyridine–sensitive current, the action potential, and the QT interval suggests that the transient outward current may be an important determinant in the relation between age and repolarization. (Circulation Research 1992;71:1390–1403)

Key Words • ventricular repolarization • epicardial myocytes • neonatal • transient outward currents

A number of currents contribute to action potential repolarization, the presence of which are species, tissue, and perhaps age specific. In canine myocardium, the voltage-activated transient outward current (I\textsubscript{to}) and the delayed rectifying outward current are held primarily responsible for repolarization\textsuperscript{1,2}; therefore, any age-dependent differences in these currents may influence overall cardiac function. I\textsubscript{to} has been recorded in atrial, ventricular, and nodal preparations.\textsuperscript{3-12} Interestingly, a large I\textsubscript{to} is present in cells isolated from canine and feline ventricular epicardium, whereas endocardial cells isolated from these animals have a greatly reduced or absent I\textsubscript{to},\textsuperscript{2,9} Activation of I\textsubscript{to} in the tissues listed above, is reflected in a large phase 1 of repolarization, which contributes to the characteristic "spike-and-dome" action potential plateau morphology. Ventricular cells that lack I\textsubscript{to} also lack a pronounced phase 1 and are characterized by a more depolarized and rounded action potential plateau.\textsuperscript{2,13} Although the kinetics of I\textsubscript{to} vary among species, the characteristic voltage-dependent activation, voltage-and time-dependent decay, interval dependence, and sensitivity to 4-aminopyridine (4-AP) are conserved and act as distinguishing characteristics of the current. These characteristics allow testing for I\textsubscript{to} in both neonatal multicellular and single-cell preparations.

The relation between repolarization measured at the level of ion channels and their expression in both the transmembrane action potential and electrocardiogram (ECG) is an area of intense interest in electrophysiology. However, there is little understanding of how changes observed at the cellular level affect overall function of cardiac repolarization. Therefore, we sought to characterize age-related changes in repolarization properties of the canine ventricle in the whole heart, isolated tissue, and single cell. Furthermore, we explored the relation between the presence of the 4-AP-sensitive ionic current, I\textsubscript{to}, in the neonatal, young, and adult heart.

Materials and Methods

Experimental Animals

Adult beagle or mongrel dogs (10 months–3 years of age) were of either sex and weighed 8–15 kg. Neonatal beagle or mongrel dogs of either sex (0–14 days of age) weighed between 350 and 1,800 g. Another group of beagles (57–64 days of age) of either sex and weighing 3–5 kg were used for some experiments. This latter group of dogs was divided into three subgroups (57–58 day-old, 60–61 day-old, and 64–68 day-old), based on developmental processes occurring in the heart, including rapid growth\textsuperscript{14,15} and sympathetic innervation.\textsuperscript{16-18}

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as well as on our ability to pinpoint the appearance of the notch in the action potential during repolarization.

**Electrocardiograms**

Lead II ECGs were recorded from conscious adult, neonatal, and young dogs by use of invasive electrodes attached to the limbs with Velcro strips and an ECG amplifier unit of variable gain at 200 mm/sec (Gould Inc., E. Rutherford, N.J.). Adult dogs were placed in a standing sling, and younger animals were gently restrained on a table for the recordings. All ECG intervals were measured (Figure 1), and the mean values for each interval in each age group were determined. The JT interval was calculated as the QT duration minus the QRS duration and includes the ST interval and T wave. To meaningfully compare age-related differences in the time course of ventricular repolarization, QT and JT intervals were corrected for heart rate (QT, and JT, respectively) by use of the formula described by Bazett and defined as

\[
QT_i = QT \div \sqrt{RR}
\]

where QT and RR are the measured values expressed in seconds. Bazett's formula was originally shown to apply to children as well as adults. Corrected intervals were averaged to obtain the mean value for each age group, and comparisons were made using the mean rate-corrected values. It is noted that ECG interval measurements based on a single lead can be confused if the instantaneous vector shifts with changes in age or tissue mass. Certainly the use of a single lead may and probably does not provide information about the longest QT interval that occurs at any age. Nevertheless, we reasoned that the concordance of changes in repolarization in ECG with those in the action potential and ionic currents would imply an association among the three.

**Multicellular Preparations**

Dogs were anesthetized with 30 mg/kg pentobarbital sodium intravenously (adult and young dogs) or intraperitoneally (neonatal dogs). The heart was rapidly removed via a lateral thoracotomy (adult dogs) or median sternotomy (young and neonatal dogs) and placed in cool Tyrode's solution containing (mM) NaCl 131, NaHCO3 18, KCl 4, NaH2PO4 1.8, CaCl2 2.7, MgCl2 0.5, and dextrose 5.5. A section of left ventricular epicardium (1–2 mm thick), located half of the way from the apex to the base and just right of the left anterior descending coronary artery, was then carefully removed with a scalpel blade. For studies of the action potential, tissues with a final dimension of 5×2 mm were first placed into a Lucite bath, epicardial side up, and then allowed to equilibrate for at least 1 hour while being

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**TABLE 1. Electrocardiographic Interval Durations**

<table>
<thead>
<tr>
<th>Age</th>
<th>n</th>
<th>RR (msec)</th>
<th>P (msec)</th>
<th>PR (msec)</th>
<th>QRS (msec)</th>
<th>QT (msec)</th>
<th>JT (msec)</th>
<th>QTc</th>
<th>JTc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>6</td>
<td>582±53</td>
<td>44±3</td>
<td>148±4</td>
<td>41±2</td>
<td>207±8</td>
<td>166±7</td>
<td>273±3</td>
<td>219±3</td>
</tr>
<tr>
<td>64–68 Days</td>
<td>3</td>
<td>357±32</td>
<td>40±0</td>
<td>117±3*</td>
<td>37±3</td>
<td>163±13</td>
<td>127±12</td>
<td>273±8</td>
<td>212±9</td>
</tr>
<tr>
<td>60–61 Days</td>
<td>3</td>
<td>350±23</td>
<td>35±3</td>
<td>100±6*</td>
<td>28±3</td>
<td>167±3*</td>
<td>140±3</td>
<td>283±8</td>
<td>238±8</td>
</tr>
<tr>
<td>57–58 Days</td>
<td>3</td>
<td>280±10</td>
<td>35±5</td>
<td>102±8*</td>
<td>35±5</td>
<td>170±0</td>
<td>135±5</td>
<td>321±6*</td>
<td>255±10*</td>
</tr>
<tr>
<td>Neonate</td>
<td>6</td>
<td>269±4*</td>
<td>36±2</td>
<td>96±3*</td>
<td>28±2*</td>
<td>168±2*</td>
<td>140±3*</td>
<td>324±2*</td>
<td>269±3*</td>
</tr>
</tbody>
</table>

\(n\), Number of dogs; QTc, rate-corrected QT interval; JTc, rate-corrected JT interval, which was calculated as the QT duration minus the QRS duration and includes the ST interval and T wave. Values are mean±SEM.

An analysis of variance and F test were performed to determine whether the sample means differed significantly. If significant differences were found, a t test with Bonferroni's procedure to correct for multiple comparisons was used to compare the values.

*\(p<0.05\) vs. adult.
superseded at a rate of 12 ml/min with Tyrode’s solution (37±0.5°C, pH 7.35±0.05). Tissues were stimulated, and action potentials were recorded as previously described. Only those experiments in which a single impalement was maintained throughout the study were included in data analysis. Measurements of maximum diastolic potential, action potential overshoot, phase 1 positive amplitude, and action potential duration (APD) at both 50% (APD50) and 90% (APD90) repolarization were made as illustrated in Figure 3A. Amplitude of the action potential plateau notch was determined by subtracting phase 1 amplitude from the overshoot amplitude (OS–P1), which corresponds to the phase 1 magnitude reported by others. Calibration and determination of the maximum slope of phase 0 of the action potential (Vmax) were performed as previously described.

For experiments using 4-AP (98% purity, Sigma Chemical Co. St. Louis, Mo.), the compound was dissolved in a modified Tyrode’s solution using low heat and vigorous stirring. Modification of Tyrode’s solution was necessary to prevent precipitation of 4-AP; 1 mM MgCl2 was included instead of 0.5 mM, and 0.33 mM Na2HPO4 was included instead of 1.8 mM. The solution had a pH of 7.35±0.05 after the addition of 4-AP. After the equilibration period and after control data were recorded, the tissue was superfused for 20 minutes with Tyrode’s solution containing 2 mM 4-AP, and action potentials and Vmax were recorded. After washout (15–30 minutes), action potentials and Vmax were again recorded.

Epicardial Myocytes

The method used for dissociation of cells from adult and neonatal ventricular epicardium was modified from that reported by Heathers et al, using CLS II-type collagenase (Worthington Biochemical Corp., Freehold, N.J.) of one of the following activities: 284, 276, 271, 231, or 198 units/mg. A 0.4–0.5 mg/ml collagenase solution was used to dissociate adult tissue, and a 0.10–0.20 mg/ml collagenase solution was used for the neonatal tissue. Protease (type XIV bacterial, Sigma) having specific activity of 5.8, 5.2, or 4.8 units/mg was added to the collagenase solution for one step. The amount of protease used per experiment was 25–35 units for adults and 9–12 units for neonates. Adult cells were stored in a 0.5 mM calcium/HEPES solution, and neonatal cells were stored in a 0.1 mM calcium/HEPES solution at room temperature for up to 12 hours.

Electrophysiology of Epicardial Myocytes

A few drops of the cell suspension were placed on a poly-l-lysine–coated glass coverslip, fit into the bottom of a 0.80-ml Lucite tissue bath, and mounted on the stage of an inverted microscope (Nikon Diaphot-TMD, Nikon Instruments, Tokyo). Cells were left to adhere to the coverslip for 5 minutes before superfusion with experimental solutions at a flow rate of 2 ml/min. A

### Table 2. Control Transmembrane Potential Characteristics in Canine Epicardium at a Basic Cycle Length of 1,300 msec

<table>
<thead>
<tr>
<th>Age</th>
<th>n</th>
<th>MDP (-mV)</th>
<th>OS (mV)</th>
<th>Vmax (V/sec)</th>
<th>APD50 (msec)</th>
<th>APD90 (msec)</th>
<th>P1 (mV)</th>
<th>OS–P1 (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>18</td>
<td>84±2</td>
<td>13±1*</td>
<td>179±11</td>
<td>101±8*</td>
<td>140±9*</td>
<td>4±1</td>
<td>9±1*</td>
</tr>
<tr>
<td>64–68 Days</td>
<td>12</td>
<td>88±1</td>
<td>15±1*</td>
<td>151±10</td>
<td>108±5*</td>
<td>152±7*</td>
<td>8±1</td>
<td>6±1*</td>
</tr>
<tr>
<td>60–61 Days</td>
<td>8</td>
<td>88±2</td>
<td>19±2</td>
<td>139±13</td>
<td>126±12*</td>
<td>168±15*</td>
<td>16±2†</td>
<td>3±1†</td>
</tr>
<tr>
<td>57–58 Days</td>
<td>7</td>
<td>85±1</td>
<td>21±2†</td>
<td>178±11</td>
<td>136±2†</td>
<td>180±16†</td>
<td>20±2†</td>
<td>1±0†</td>
</tr>
<tr>
<td>Neonate</td>
<td>20</td>
<td>83±1</td>
<td>25±1†</td>
<td>185±12</td>
<td>168±5†</td>
<td>221±6†</td>
<td>23±2†</td>
<td>0±2†</td>
</tr>
</tbody>
</table>

nP, Number of tissue sections; MDP, maximum diastolic potential; OS, action potential overshoot; Vmax, maximum slope of phase 0 of the action potential; APD50 and APD90, action potential duration at 50% and 90% repolarization, respectively; P1, phase 1 amplitude above 0 mV reference line. Values are mean±SEM.

An analysis of variance and F test were performed to determine whether the sample means differed significantly. If significant differences were found, a t test with Bonferroni’s procedure to correct for multiple comparisons was used to compare the values.

*p<0.05 vs. neonate.
†p<0.05 vs. adult.
HEPES bath solution containing (mM) NaCl 134, KCl 4, MgCl\textsubscript{2} 0.5, CaCl\textsubscript{2} 2, glucose 6, and HEPES 10 (pH 7.4, 37°C) was used in all single-cell experiments. Glass microelectrodes filled with 3 M KCl (tip resistances, 30–70 MΩ) were used to record transmembrane potentials (Axoclamp-2A amplifier with an HS-2L gain ×0.1 headstage, Axon Instruments, Foster City, Calif.). Cells were allowed to recover after impalement, and data were only analyzed from cells that could maintain a stable resting and action potential upon removal of all holding current. The cells were stimulated at a basic cycle length (BCL) of 1,300 msec, with brief current pulses of suprathreshold amplitude. Current and voltage were displayed on a dual-beam storage oscilloscope and recorded with Polaroid film.

**Whole-Cell Patch-Clamp Recordings**

Currents were recorded from myocytes with a continuous-clamp voltage-clamp technique in the whole-cell configuration by use of an Axopatch-1D amplifier (CV-4 1/100 or CV-3 0.1/100 headstage) and an Axon TL-1 DMA analog/digital converter (Axon Instruments). Suction pipettes made of borosilicate glass (1.5 mm o.d. and 0.86 mm i.d., Sutter Instrument Co., Novato, Calif.) were heat-polished before use. To isolate potassium currents, pipettes were filled with a solution containing (mM) potassium aspartate 125, KCl 20, EGTA 10, ATP (magnesium salt) 10, MgCl\textsubscript{2} 1, and HEPES 5 (pH 7.3). The electrode resistances were 1–2 MΩ for the adult cells and 2.5–5 MΩ for the neonatal cells when filled with the internal solution.

Capacitance due to the pipette was electronically compensated. In the neonatal cells, cell capacitance and series resistance (up to 100%) were also electronically compensated. For adult cells, capacitive compensation was not complete, but 100% series resistance compensation was possible. The time constant describing decay of the capacitive transient was 0.41±0.1 msec in adult cells (n=17) and 0.18±0.02 msec in neonatal cells (n=6). Series resistance was determined from Axopatch meter readings and was found to equal 1.5–2 times the pipette resistance.\textsuperscript{25} The seal resistance between the electrode tip and the cell membrane was >10 GΩ for all experiments.

To quantify I\textsubscript{so}, it was necessary to minimize other contaminating currents. The sodium current was largely suppressed by the addition of 30 μM tetrodotoxin (TTX) to the superfusion solution, and in some cases, a holding potential of −60 mV was used. The calcium current was suppressed by the addition of 2 mM manganese to the superfusion solution, which might produce a slight positive shift in I\textsubscript{so} inactivation.\textsuperscript{2} The calcium-activated transient outward current was blocked by 2 mM manganese as well as 10 mM EGTA in the pipette solution.\textsuperscript{2} The ATP-dependent potassium current remained inactivated because of the presence of 10 mM ATP (magnesium salt) in the pipette solution. Contamination due to the delayed rectifier potassium current was minimal, since this current activates more slowly.

### Table 3. Transmembrane Potential Characteristics in Canine Epicardium Before and During 4-Aminopyridine Superfusion

<table>
<thead>
<tr>
<th>Age</th>
<th>n</th>
<th>Superfusion</th>
<th>MDP (mV)</th>
<th>OS (mV)</th>
<th>V\textsubscript{max} (V/sec)</th>
<th>APD\textsubscript{50} (msec)</th>
<th>APD\textsubscript{90} (msec)</th>
<th>P1 (mV)</th>
<th>OS−P1 (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>10</td>
<td>Control</td>
<td>86±2</td>
<td>14±1</td>
<td>183±18</td>
<td>102±5</td>
<td>141±7</td>
<td>5±1</td>
<td>9±1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-AP</td>
<td>85±2</td>
<td>18±1*</td>
<td>157±14</td>
<td>121±8*</td>
<td>178±9*</td>
<td>14±2*</td>
<td>4±1*</td>
</tr>
<tr>
<td>64–68 Days</td>
<td>12</td>
<td>Control</td>
<td>88±1</td>
<td>15±1</td>
<td>151±10</td>
<td>108±5</td>
<td>152±7</td>
<td>8±1</td>
<td>6±1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-AP</td>
<td>91±1</td>
<td>22±1*</td>
<td>151±10</td>
<td>131±5*</td>
<td>180±6*</td>
<td>19±1*</td>
<td>3±1*</td>
</tr>
<tr>
<td>60–61 Days</td>
<td>8</td>
<td>Control</td>
<td>88±2</td>
<td>19±2</td>
<td>139±13</td>
<td>126±12</td>
<td>168±15</td>
<td>16±2</td>
<td>3±1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-AP</td>
<td>89±2</td>
<td>21±2</td>
<td>132±8</td>
<td>127±8</td>
<td>172±12</td>
<td>20±2</td>
<td>1±1</td>
</tr>
<tr>
<td>57–58 Days</td>
<td>7</td>
<td>Control</td>
<td>85±1</td>
<td>21±2</td>
<td>178±11</td>
<td>136±12</td>
<td>190±16</td>
<td>20±2</td>
<td>1±0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-AP</td>
<td>86±2</td>
<td>23±2</td>
<td>148±16</td>
<td>142±4</td>
<td>197±4</td>
<td>23±2</td>
<td>1±0</td>
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<td>Neonate</td>
<td>12</td>
<td>Control</td>
<td>84±1</td>
<td>23±2</td>
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<td>180±6</td>
<td>233±7</td>
<td>22±1</td>
<td>0±1</td>
</tr>
<tr>
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<td>4-AP</td>
<td>86±2</td>
<td>23±2</td>
<td>193±18</td>
<td>175±6</td>
<td>232±8</td>
<td>23±1</td>
<td>0±1</td>
</tr>
</tbody>
</table>

\textsuperscript{n} Number of tissue sections; MDP, maximum diastolic potential; OS, action potential overshoot; \textit{V}_{\text{max}}, maximum slope of phase 0 of the action potential; APD\textsubscript{50} and APD\textsubscript{90}, action potential duration at 50% and 90% repolarization, respectively; P1, phase 1 positive amplitude; 4-AP, 4-aminopyridine. Values are mean±SEM.

\textsuperscript{*}p<0.05 vs. control of same age group.

\textsuperscript{†}p<0.05 vs. neonate (control).
The capacitive currents, although 4-AP block of TTX-insensitive inward currents may cause slight underestimation of $I_{i,0}$ amplitude. In our experiments, the amplitude of $I_{i,0}$ was measured as the difference between the peak of the 4-AP-sensitive current and the current at the end of the clamp step. Time to peak of the 4-AP-sensitive current was measured as the time from the beginning of the depolarizing clamp step to the peak of the difference current. The time course of decay of the 4-AP-sensitive current was determined by fitting the decay from the peak to 100 msec after the peak, using CLAMPFIT, version 5.5.

The outward current found in some neonatal myocytes was insensitive to 4-AP (see “Results”) and could not be analyzed as a difference current. Therefore, the peak of the neonatal current was measured as the difference between the peak current and the holding current level, or baseline. The amount of current decay during a maintained clamp step was measured as percentage of the peak current remaining at a given time after the peak. For the adult $I_{i,0}$, the time course of decay of the 4-AP-sensitive transient outward current was best described by the single exponential function. This is in agreement with the equations used to fit decay of $I_{i,0}$ in other adult cardiac preparations.

Statistical Analysis

For multiple comparisons, an analysis of variance was used, followed by Bonferroni’s test. A matched-pair $t$ test was used to test the response of action potentials and currents to drug intervention (e.g., 4-AP). A two-sample $t$ test was used in cases in which a single comparison between two independent groups was made. Because of the differing proportions of animal strains (beagles and mongrels) in the different age groups, the influence of strain on the presence or absence of the different outward currents was explored by use of a $\chi^2$ test for independent samples. For all comparisons, differences were declared significant at $p<0.05$. All values are reported as mean±SEM.

Electrocardiograms

Lead II ECGs were recorded from conscious dogs to determine whether age-related differences occur in the currents.
time course of ventricular repolarization (Figure 1). Neonatal ECGs differ significantly from those recorded from the adults for every measured interval except the P wave (Table 1). Although the QT interval is significantly shorter in the neonates and 60–61-day-old animals as compared with the adults, the rate-corrected parameter QTc is significantly longer in the neonates and 57–58-day-old animals. Similarly, the rate-corrected parameter JT, is longer in the neonates and 57–58-day-old animals than in the adults, suggesting a significantly longer duration of ventricular repolarization in younger animals.

Recordings From Multicellular Preparations

Representative tracings of epicardial action potentials recorded under control conditions from canine hearts of different ages are shown in Figure 2. Mean action potential characteristics are presented in Table 2. Figure 3A indicates the points at which the action potential measurements were made. As for the ECG recordings, the largest differences in action potential repolarization are seen when comparing potentials of neonates to adults (Figure 3B). The potentials recorded from 57–61-day-old animals form an intermediate group differing from both the neonatal and adult epicardial action potentials. OS–P1 mean values calculated for the neonates up through the 61-day-old animals differ from the adult mean value. In addition, although tissues from neonatal and 57–61-day-old hearts were paced at BCLs of 3,000 and 5,000 msec, the slower pacing rates did not reveal a more pronounced phase 1 repolarization or spike-and-dome plateau morphology in these age groups.

The action potential significantly decreases in duration with increasing age. Mean neonatal APD50 and APD90 values differ from adult mean values; mean APD50 and APD90 values obtained from animals aged 60 days through adult differ from mean neonatal values; and values of APD50 and APD90 obtained from 57–58-day-old animals differ from both adult and neonatal mean values.

Since the occurrence of phase 1 repolarization and the spike-and-dome morphology in adult epicardial fibers is characteristic of the presence of Ito1,2,5,13,30 our data suggested that neonatal fibers might lack this current. Therefore, we superfused tissue sections with 4-AP (2 mM), a known blocker of Ito.2 As shown in Table 3, 4-AP significantly increased the action potential overshoot and phase 1 amplitude in fibers from animals aged 64 days through adult. Furthermore, 2 mM 4-AP resulted in a significant but reversible increase in both APD50 and APD90 in the 64–68-day-old and adult epicardium. In marked contrast, there were no significant changes in any potential characteristics produced by superfusion of 2 mM 4-AP in neonatal through 61-day-old fibers.

A separate group of tissues were paced at a BCL of 300 msec to further explore developmental differences in repolarization. As previously reported, rapid pacing results in a decreased APD in both adult and neonatal fibers.23 However, the effect of rapid pacing on the action potential plateau proved to be different between the age groups. Pacing the adult epicardium at a BCL of 300 msec produced a significant increase in phase 1 amplitude (from 4±1 mV at a BCL of 1,300 msec to 11±1 mV at a BCL of 300 msec, n=8) and a significant decrease in OS–P1 (9±2 mV at a BCL of 1,300 msec to 4±1 mV at a BCL of 300 msec), resulting in a reduction of the spike-and-dome–shaped plateau. Pacing neonatal epicardial fibers (n=8) at a BCL of 300 msec resulted in no change in action potential plateau parameters. These findings further suggest that the presence of rate-dependent Ito in adult tissue and its absence in neonatal tissue may contribute to differences in repolarization. This hypothesis was further explored with whole-cell voltage-clamp studies of myocytes dispersed from the epicardium.

Recordings From Single Myocytes

Healthy calcium-tolerant cells could consistently be isolated from the ventricular epicardium of adult and
neonatal dogs. Neonatal cells are significantly smaller in size (length, 36±6 μm; width, 6±0 μm, n=8) than adult cells (length, 140±8 μm; width, 21±3 μm, n=6) and have a significantly reduced cell capacitance (15±4 pF, n=34) as compared with adults (102±8 pF, n=38). Typical transmembrane action potentials recorded from myocytes using 3 M KCl–filled microelectrodes are shown in Figure 4. Action potentials recorded from single adult myocytes differ from those recorded from adult fibers for all characteristics except maximum diastolic potential (Table 4). In particular, the adult myocyte action potential has a more pronounced spike-and-dome-shaped plateau and a significantly longer duration. The potentials recorded from neonatal myocytes have a significantly larger overshoot and thus OS–P1 compared with potentials recorded from neonatal fibers. Despite this, a spike-and-dome-shaped plateau did not become apparent in the dissociated neonatal myocyte (Figure 4). As for adults, potentials recorded from neonatal myocytes were longer in duration compared with the potentials of fibers. Regardless of the changes due to the type of preparation, potentials recorded from both adult and neonatal myocytes confirm that age-related differences seen in repolarization (e.g., overshoot, phase 1 amplitude, OS–P1, and APD90) of intact tissue are conserved at the single-cell level (Table 4).

**Transient Outward Current**

In all adult myocytes (n=27), I_{o1} could be recorded during clamp steps to various test voltages. The I_{o1}, as characterized below, is similar to that previously reported. The 4-AP–sensitive current in adult cells, I_{o1}, is characterized by rapid activation and decay during depolarized clamp steps (Figure 5). The threshold for current activation was between −30 and −20 mV (Figure 5A). Peak currents elicited with less depolarized steps (test voltages $V_T$, from −30 to 0 mV) may be slightly underestimated because of contaminating inward currents (see “Discussion”). Mean time to peak values for the adult I_{o1} range from 2.5 to 3.3 msec and decrease with the more positive $V_T$; however, this relation is not statistically significant (Figure 5B). Mean values of time constants of decay of I_{o1} ranged between 5.5 and 6.8 msec and did not vary significantly with $V_T$ (Figure 5C).

In 63 neonatal cells studied using similar clamp protocols, none exhibited an I_{o1} of the type found in the adult myocytes. In 17 cells (37°C) in which health was verified by the presence of large inward currents seen in control solutions (Figure 6A), the currents recorded in the presence of 4-AP, TTX, and manganese (Figure 6C) were subtracted from the currents recorded in the absence of 4-AP (Figure 6B); a 4-AP–sensitive transient outward current was not revealed (Figure 6E). Additional experiments were performed in the 63 neonatal cells studies by use of various holding potentials ($V_h$, from −80 to −40 mV), $V_T$ (to +120 mV), clamp intervals (3–20 seconds), clamp durations (200–10,000 msec), and temperatures (30° and 37°C) in an effort to reveal an adult-type I_{o1}. All efforts were unsuccessful. Therefore, it appears that neonatal canine epicardial myocytes do not exhibit a 4-AP–sensitive I_{o1} similar to that found in 100% of the adult epicardial myocytes. This conclusion is consistent with the lack of both phase 1 repolarization and a spike-and-dome-shaped action potential plateau in the neonatal myocyte and fiber.

**Neonatal Outward Current**

A rapidly activating, slowly decaying outward current was observed in 23% (17 of 75) of all neonatal cells studied (Figure 7A). A similar current was never observed in adult myocytes (n=76). The presence of this current could not statistically be correlated with strain.
sex, or specific neonatal age (between 1 and 14 days) of the heart from which it was disaggregated. An analysis of this outward current, unique to neonatal cells, and its similarity to other outward currents are reported in the following paragraphs.

To determine the peak current–voltage relation of the neonatal current, the membrane was clamped to various $V_t$ from a $V_h$ of $-80$ mV using the same protocol as described previously for the adult $I_{tot}$. The neonatal current has a sigmoidal onset of activation (Figure 7C) and rises quickly to a peak (data not shown). However, unlike the $I_{tot}$, the neonatal current does not rapidly decay to baseline during maintained depolarization (Figures 7A, 7D, and 7E). In neonatal cells, the peak density of the outward current increases with increasing $V_t$, much like the adult $I_{tot}$. In fact, the density of the peak neonatal current is not significantly different from the peak density of the adult $I_{tot}$ at any $V_t$ (Figure 7B). In contrast, density of the outward current at the end of the clamp step in adult cells is significantly less than the density of the neonatal current at a similar time point, for all levels of $V_t$ tested. Furthermore, density of neonatal currents at the end of the clamp step do not differ from the peak density of neonatal currents at any $V_t$ (Figure 7B), confirming that decay of the neonatal current is less rapid and less complete as compared with decay of $I_{tot}$ during maintained clamp steps.

One characteristic of the adult $I_{tot}$ is its frequency dependence, or inability to recover quickly after activation; $I_{tot}$ increases rapidly with frequency (range, 20–75 pA), reaches a peak rapidly, and slowly decays during the clamp step. This type of difference current was observed in only four of 10 neonatal cells. On average, the difference current density in the neonatal cells was significantly less than the density of the neonatal current recorded at a BCL of 3,000 msec, confirming the lack of interval dependence.

The neonatal current also differed from the adult $I_{tot}$ in its sensitivity to 4-AP (Figure 9). There was no significant decrease in mean peak neonatal current in seven neonatal cells superfused with 4-AP (450±32 pA for control values, 420±24 pA for values after the administration of 4-AP). However, in three of seven cells studied, a small 4-AP difference current was...
revealed (Figure 9C). The difference current varied among these cells, reaching a peak current amplitude of 23–109 pA (2–6 pA/pF) in 17–45 msec after the start of depolarization. 4-AP did not abolish the outward current in any neonatal cell. Therefore, the neonatal current cannot be classified as a "4-AP-sensitive" outward current, in contrast to the I_{o1} described in adult cells.

The peak amplitude of I_{o1} in adult cells is dependent on holding potential. In four adult and three neonatal cells, membrane currents were recorded from V_h values of −80 and −40 mV in the same cell (Figure 10). The adult I_{o1} recorded from a V_h of −40 mV (Figure 10B) is significantly reduced at all levels of V_h, when compared with the currents recorded at a V_h of −80 mV (Figure 10A). Specifically, at a depolarized clamp step (V_h = +50 mV), the adult I_{o1} mean peak density was 24±4 pA/pF when V_h was −80 mV and 5±0.4 pA/pF when V_h was −40 mV. Unlike the adult current, the amplitude of the neonatal current is not affected by V_h (Figure 10); e.g., at V_h of +50 mV, the current is 28±10 pA/pF when V_h is −80 mV and the current is 25±8 pA/pF when V_h is −40 mV.

Discussion

Our experiments demonstrate that there are age-related differences in ventricular repolarization manifested in the ECG, the epicardial action potential, and repolarizing currents. In fact, one of the major repolarizing currents occurring in adult epicardial cells is lacking in neonatal cells. In addition, a unique outward current was recorded from neonatal epicardial myocytes, suggesting an altered expression of ion channels with development.

Electrocardiograms

The ECGs reported here had mean durations of P, PR, QRS, and QT intervals and complexes that were within the range reported in previous studies of dogs and humans. Our results clearly indicate that, when differences in heart rate are accounted for, the neonates and 57–58 day-old animals require a greater amount of time for complete ventricular repolarization than do adults. The lack of significant differences in QT and JT, among the 60–68-day-old and adult dogs suggests that developmental maturation of repolarization may be completed by the 60th postnatal day.

Major factors influencing the duration and polarity of ECG intervals and complexes that could contribute to the changes observed in QT and JT, with age include the mass of cardiac tissue that is activated, the pattern of activation of the cardiac chambers, and properties of the underlying action potentials. More specifically, in chambers of small mass the amount of tissue excited would be smaller; the distance the wave front travels would be less; and if mature conduction pathways exist, the ECG complexes would be expected to have shorter total duration as compared with recordings from large chambers. An age-related difference in the pattern of ventricular activation could likewise contribute to differences observed in QT and JT durations and morphologies. Body surface maps of cardiac activity suggest a right ventricular dominance of activation in hearts of human infants, which is different from the left ventricular dominance of older human hearts. Difference in ventricular dominance causes a shift in mean ECG vectors and may contribute to some of the developmental differences observed in canine ECGs. Finally, developmental differences in the ECG descriptors of repolarization may be due to a summation of changes occurring in the underlying action potentials. A rigorous analysis of all factors contributing to the observed changes in ECG intervals are beyond the scope of this study. We concentrated our attention on differences in

![Figure 8](http://circres.ahajournals.org/doi/10.1161/01.RES.71.6.1398)
canine ventricle\textsuperscript{42} are characteristically similar to those recorded from adult canine endocardium.\textsuperscript{10} Thus, repolarization in ventricular endocardium does not appear to change dramatically with development. In contrast, repolarization of canine epicardium changes with age. Therefore, we focused on determining the age-related differences in action potential repolarization of epicardial tissue.

Values for maximum diastolic potential and $V_{\text{max}}$ of the adult action potentials from our experiments do not differ from values reported for action potentials recorded from other canine epicardial preparations.\textsuperscript{10,33,41} Although the values we report for adult APD are somewhat less than those reported for right ventricular epicardium at a BCL of 2,000 msec,\textsuperscript{10,33} they are within the range reported in a study of left ventricular epicardium at a BCL of 1,300 msec.\textsuperscript{43} APD is influenced by time of tissue equilibration, temperature, superfusate composition, potassium accumulation, and site of the tissue,\textsuperscript{44,45} variables that make it difficult to compare APD among studies. Because of these inconsistencies, the important factor in a study such as ours is that, under identical experimental conditions, there is a difference in APD among age groups.

Phase 1 repolarization in our adult action potential recordings is similar to that reported for adult canine and feline epicardium.\textsuperscript{10,30,33,41} Interestingly, the phase 1 repolarization in adult epicardium, and lack thereof in neonatal epicardium (Figure 2), is strikingly similar to the differences between epicardial and endocardial action potentials seen in other preparations of adult tissue.\textsuperscript{9,10,30,41,46} In fact, the plateau parameters reported for canine endocardium\textsuperscript{10} are similar to the plateau parameters we report for neonatal epicardium (Table 2). It is believed that differences in phase 1 repolarization between adult epicardial and endocardial fibers and myocytes are primarily due to the presence of $I_{\text{ong}}$ in the epicardium and absence of the current in the endocardium.\textsuperscript{9} Thus, a developmental increase in $I_{\text{ong}}$ may play a role in the appearance of phase 1 repolarization with age (Figure 2). Age-dependent increases in

![Figure 9](http://circres.ahajournals.org/)

**Figure 9.** *The effects of 4-aminopyridine (2 mM) on the neonatal outward current. Membrane currents were recorded from a neonatal epicardial myocyte in response to a 200-msec clamp step to a test voltage of +40 mV from a holding potential of -80 mV at 37°C. The cell was superfused with a HEPES solution containing 30 µM tetrodotoxin and 2 mM manganese (panel A), before the addition of 2 mM 4-aminopyridine to the bath solution for an additional 5-minute superfusion (panel B). The 4-aminopyridine difference current was obtained (panel C). Vertical calibration is 42 pA, and horizontal calibration is 20 msec. Cell 90524 (15 pF).*

action potentials and ionic currents underlying the ECGs, as discussed below.

**Recordings From Multicellular Preparations**

Differences in the repolarization of epicardium and endocardium are well documented in adult canine ventricle.\textsuperscript{10,33,41} Action potentials recorded from fetal

![Figure 10](http://circres.ahajournals.org/)

**Figure 10.** *Superimposed tracings of adult (left tracings) and neonatal (right tracings) membrane currents recorded at different holding potentials in the same cell. Currents were recorded in response to 200-msec clamp steps to test voltages between -30 and +60 mV from a holding potential of either -80 mV (panel A) or -40 mV (panel B). All currents were recorded in a HEPES bath solution containing 30 µM tetrodotoxin and 2 mM manganese at 37°C. Vertical calibrations are 400 pA (adult) and 100 pA (neonate), and horizontal calibrations are 8 msec (adult) and 20 msec (neonate). Cell 90419 (88 pF) and cell 90521 (15 pF).*
phase 1 repolarization have also been observed in action potentials recorded from rat myocardium,47 rabbit papillary muscles,48 canine Purkinje fibers,49 canine atrium,50 and human atrium.51

Two distinguishing characteristics of \(I_{\text{tot}}\) are rate dependence and 4-AP sensitivity. We tested the influence of these variables on phase 1 repolarization and plateau morphology in epicardial action potentials. In adult tissues, the amplitude of phase 1 is greatly diminished by rapid pacing, as seen by others.31,33,34,51,52 Similar pacing of neonatal tissue results in no significant changes in action potential plateau. Other studies have shown that action potentials from neonatal hearts lacking the rapid phase 1 repolarization also lack rate-dependent changes in action potential plateau voltage and morphology.48,51

4-AP superfusion of epicardial tissue from adult and 64–68-day-old hearts resulted in a significant increase in phase 1 amplitude. A similar increase in phase 1 amplitude has been observed in other adult tissues.10,48,49,51 4-AP superfusion did not change phase 1 of action potentials recorded from epicardial tissue of neonatal through 61-day-old animals. This is important because it clearly delineates age-related differences in a 4-AP effect, further suggesting a difference in the potassium currents.

To directly compare the amplitude and kinetics of ionic currents in neonatal and adult tissue, we developed a method of dissociating myocytes from the epicardium. Action potentials recorded from these myocytes established the viability of the cells and demonstrated that developmental differences observed in multicellular preparations were conserved at the single-cell level.

Adult and neonatal myocytes retained a well-polarized maximum diastolic potential after dissociation, which did not differ significantly from values recorded in the respective multicellular preparations. However, both the adult and neonatal myocytes had significantly increased OS–P1 and APD values as compared with the multicellular preparations. The increased OS–P1 in neonatal myocytes was not due to the appearance of rapid phase 1 repolarization but rather to an increase in the action potential plateau slope, which became apparent after dissociation. Importantly, there was no change in the age-related presence of phase 1 repolarization in epicardial myocytes after the cell dissociation procedure. All action potentials recorded from adult epicardial myocytes demonstrated a large phase 1 repolarization (see also Reference 13), and potentials from neonatal epicardial myocytes did not. Therefore, the cell dissociation procedure itself does not result in loss or appearance of the spike-and-dome plateau morphology.

**Transient Outward Current**

Transient outward currents recorded from adult epicardial myocytes in our experiments were voltage dependent and Ca\(^{2+}\) independent and had a linear current density–voltage relation and a rapid time to peak; these characteristics are similar to those reported previously for canine \(I_{\text{tot}}\).1,13 It should be noted that complete elimination of contaminating inward currents was not achieved in some of our experiments. Because the focus of this study was on differences between adult and neonatal cells, it was essential to use the same solutions for both preparations. Thus, physiological sodium containing solutions along with multiple ion channel blockers were chosen for all recordings to ensure the viability of neonatal cells (as discussed below).

Therefore, the presence of \(I_{\text{tot}}\) in our preparation of adult epicardial myocytes is consistent with both the presence of a large phase 1 repolarization and the 4-AP effects that were observed in action potential recordings from adult epicardial tissue. In this study, using similar protocols, we never observed a measurable 4-AP–sensitive \(I_{\text{tot}}\) in the neonatal myocyte. Lack of a recordable 4-AP difference current in the neonatal myocyte may have been due to an age-related difference in reactivation characteristics of \(I_{\text{tot}}\), resulting in the inability to record the current with the clamp interval protocol used. Studies of action potentials recorded from human atrial tissue suggest that there is an age-related delay in reactivation of the \(I_{\text{tot}}\).51 Because of this, protocols at various clamp intervals, ranging from 1 to 20 seconds, were used in neonatal cells in an effort to observe \(I_{\text{tot}}\). All efforts were unsuccessful.

One might argue that \(I_{\text{tot}}\) was not recorded in the neonatal myocytes because its activation and inactivation kinetics were too rapid to enable its isolation from tracings of total membrane current. However, no currents were revealed in the 4-AP difference current tracings, suggesting that not even an extremely rapid \(I_{\text{tot}}\) is present in the neonatal myocytes. It is possible that the neonatal \(I_{\text{tot}}\) is very rapid and also insensitive to 4-AP, thus preventing its appearance in the 4-AP difference current tracings. However, studies of the effects of 4-AP on preparations of rat ventricle53 and human atrium54 suggest that sensitivity to 4-AP is present as soon as a rapidly decaying \(I_{\text{tot}}\) can be observed. Alternatively, because residual inward currents persisted in the continued presence of TTX and manganese (Figure 6), there may have been difficulty in identifying very small 4-AP–sensitive currents using our difference current approach. Nevertheless, if the density of transient outward currents in neonatal myocytes were on the order of the density of 4-AP–sensitive currents in adult cells, we should have readily resolved these transient currents (150–200 pA at positive \(V_c\)). Others have reported that a developmental increase in the amplitude of \(I_{\text{tot}}\) is contributed to by increased selectivity of the channel for K\(^{+}\) ions.53 Although reversal potentials were not determined in our study, various levels of \(V_c\) and \(V_e\) were used to increase the likelihood of recording an \(I_{\text{tot}}\) if such a current were present in the canine neonatal myocyte.

It is possible that lack of \(I_{\text{tot}}\) in neonatal myocytes is secondary to damage done to the channel during the dissociation procedure. However, this explanation is unlikely, since TTX- and manganese-sensitive inward currents were apparent in these neonatal myocytes, even though \(I_{\text{tot}}\) was not, indicating that channels in the cell membrane were not damaged by the dissociation procedure. Finally, the lack of phase 1 repolarization as well as the 4-AP insensitivity in action potentials recorded from neonatal epicardial fibers is consistent with the finding that \(I_{\text{tot}}\) is absent in the myocyte.

**Characteristics of the Neonatal Outward Current**

In response to depolarizing clamp steps, a unique outward current was recorded in 23% of the neonatal
epicardial myocytes. Unlike the adult $I_{\text{to}}$, the neonatal current showed little decay during a sustained depolarizing pulse and was not significantly decreased by rapid pacing or by 4-AP. Although 40% of neonatal cells showed a small degree of inhibition in response to 4-AP superfusion, in no experiment did 4-AP totally abolish or even greatly diminish the amplitude of the neonatal current. This is dramatically different from the effects of 4-AP on adult $I_{\text{to}}$ in these experiments and those reported by others.\textsuperscript{2,3,9,13,20} Furthermore, even the 4-AP difference current obtained from these few cells differed from the adult 4-AP-sensitive $I_{\text{to}}$ in that it was of low amplitude and showed little decay during the maintained clamp step.

Two types of 4-AP block of adult transient outward current have been described: one mechanism of 4-AP block occurs during depolarizing steps, when the membrane channel is in the open state; the other means of 4-AP block involves direct interference with the inactivation process.\textsuperscript{54,55} If 4-AP binds to a site on or near the inactivation gate in adult cells and if the inactivation gate is not present or nonfunctional in neonatal cells, then little or no 4-AP binding should occur in the neonate. Thus, it is possible that a developmental difference in inactivation could explain the differences in 4-AP sensitivity, decay process, and response to rapid pacing observed in the comparison of the adult $I_{\text{to}}$ and the neonatal outward current.

Although experiments to determine the charge carrier of this neonatal outward current were not performed, a number of factors suggest that the charge carrier is predominantly potassium. The solutions used in the voltage-clamp experiments were specifically selected to isolate potassium currents, and the current shows similarities to other cardiac potassium currents in time dependence, voltage dependence, and sigmoidal onset of activation. In addition, the neonatal outward current is strikingly similar to both a human potassium channel clone recently expressed in murine LtK cells\textsuperscript{56} and a unique potassium current in rat atrial cells.\textsuperscript{57}

Another ion that could carry outward current, with depolarizing steps, is chloride. Although it is impossible to rule out from our experiments, we believe it unlikely that the chloride ion is the charge carrier. A chloride current ($I_{\text{Cl}}$) with characteristics similar to the neonatal current has never been described in cardiac preparations. An $I_{\text{Cl}}$ that is ubiquitous in the heart is isoproterenol activated and time independent,\textsuperscript{58,59} characteristics that make it unlikely to be the observed neonatal outward current. Recently, a Ca$^{2+}$-activated $I_{\text{Cl}}$, similar to the calcium-activated transient outward current, has been described in ventricular myocytes.\textsuperscript{60} However, care was taken in the experiments using neonatal epicardial myocytes to block Ca$^{2+}$-activated currents (2 mM manganese, 10 mM EGTA, and no isoproterenol), making it unlikely that the Ca$^{2+}$-activated $I_{\text{Cl}}$ was recorded.

A final possibility is that the outward current described here actually reflects two or more superimposed currents, e.g., a steady time-independent outward current in the presence of a slowly increasing inward current. The superimposed currents would have to be voltage activated, temperature dependent, and not blocked by TTX, manganese, 4-AP, or ATP. A TTX-insensitive inward current superimposed on a time-independent $I_{\text{Cl}}$ is one possibility. However, the inward current would have to increase in amplitude as $V$, increased to account for the observed voltage-dependent current decay, an unlikely situation because of the less depolarized reversal potentials of inward currents. Therefore, it is most likely that the neonatal outward current is largely carried by a single ion, namely the potassium ion.

**Physiological Significance of the Neonatal Outward Current**

It is not yet understood what role the neonatal current plays in repolarization. It seems likely that a sustained current of such large amplitude would reduce the APD of neonatal epicardial cells. However, action potentials recorded from neonatal myocytes and fibers had long durations. In recording action potentials from four myocytes, there was a 35% chance (0.77\textsuperscript{4}) that action potentials with short durations were overlooked. However, there was only a 0.5% chance (0.77\textsuperscript{4}) of overlooking a short duration action potential in recordings made from epicardial fibers ($n=20$). In other words, based on the number and homogeneity of fibers studied, there is a very low probability that neonatal action potentials having short durations were present in the tissue and missed in this study. It is possible that the homogeneity of action potentials recorded from fibers may be due to electronic coupling among cells.\textsuperscript{61} The majority of which had long APDs. Therefore, although myocytes having shorter APDs may be present, the influence they have on repolarization of the intact tissue is unclear.

An outward current, distinct from both $I_{\text{to}}$ and the delayed rectifier potassium current, was recently observed in myocytes isolated from adult rat atria.\textsuperscript{59} The kinetic properties of this atrial current are remarkably similar to our neonatal outward currents and to several cloned potassium channel currents.\textsuperscript{62} The atrial $K^+$ current is voltage dependent, is characterized by rapid activation, and shows little decay during a sustained clamp step.\textsuperscript{57} However, unlike the canine neonatal current, 1–5 mM 4-AP produces open channel block of the outward current in rat atrial myocytes.\textsuperscript{57}

In conclusion, using ECG, multicellular, and single-cell recordings, we have shown that age-related changes occur in the voltage–time course of canine ventricular repolarization. Neonates and 57–58-day-old animals have a longer, more depolarized voltage–time course of repolarization when compared with adults. Our studies suggest that an absence of adult-type $I_{\text{to}}$ in the youngest animals contributes to these differences in repolarization. Additional differences in the currents responsible for repolarization are suggested by the presence of a unique outward current in the neonatal cells.

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Age-related appearance of outward currents may contribute to developmental differences in ventricular repolarization.
C D Jeck and P A Boyden

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