Developmental Changes in the Electrophysiological Properties and the \( \beta \)-Adrenergic Receptor-Effecter Complex in Atrial Fibers of the Canine Coronary Sinus

Evelyn M. Horn, Nancy J. Johnson, John P. Bilezikian, and Michael R. Rosen

\( \beta \)-Adrenergic stimulation induced delayed afterdepolarizations and triggered activity in atrial fibers of adult but not young canine coronary sinus. However, sensitivity to \( \beta \)-adrenergic agonists with respect to maximum diastolic potential was identical at both ages, and delayed afterdepolarizations and triggered activity did occur in response to ouabain. Age-dependent lengthening of the action potential duration and plateau also were seen and were greater in the adult than the young. \( \beta \)-Adrenergic receptor density and affinity and the stimulatory guanine nucleotide regulatory protein \( (G_s) \) were similar in adult and young tissues. In contrast, the inhibitory guanine nucleotide regulatory protein \( (G_i) \) was 2.5-fold greater in adult (15 fmol/mg) than in young (6.0 fmol/mg) tissues. Basal- and forskolin-stimulated adenylate cyclase activities were greater in adult than young coronary sinus although the increment in isoproterenol-stimulated adenylate cyclase activity in young tissue was greater when compared either with basal levels or expressed as a percentage of maximal catalytic activity. Both the traditional effector pathway of \( \beta \)-adrenergic action, involving the stimulation of adenylate cyclase activity, and developmental changes in the action potential plateau may contribute to the developmental changes in delayed afterdepolarizations and triggered activity. (Circulation Research 1989;65:325–333)

During the course of development, the specialized conducting system of the canine ventricle undergoes changes in transmembrane potential, in impulse initiation and conduction, and in responsiveness to autonomic agents.1-5 The present study was designed to investigate developmental changes in the transmembrane potential and autonomic modulation of atrial fibers in the canine coronary sinus. This tissue was selected because of its importance as a potential source of cardiac arrhythmias,6 which are important modulators of cardiac rhythm and arrhythmias.

We compared the transmembrane potential, delayed afterdepolarizations, and triggered activity in coronary sinus tissue from young and adult canine hearts as well as their effects on \( \beta \)-adrenergic catecholamines. In addition, developmental changes in the responsiveness of coronary sinus to \( \beta \)-adrenergic catecholamines were studied at the level of the \( \beta \)-adrenergic receptor complex, including the receptor itself, its associated guanine nucleotide regulatory proteins, and adenylate cyclase activity.

Materials and Methods

Electrophysiological Techniques

We anesthetized adult (>1 year old) and young (0–73 days old) mongrel dogs with sodium pentobarbital, 30 mg/kg i.v. The hearts were removed via a right lateral thoracotomy or a midline sternotomy (young) and immediately immersed in cold Tyrode’s solution containing (mM) NaCl 131, NaHCO\(_3\) 18, KCl 4, NaH\(_2\)PO\(_4\) 1.8, MgCl\(_2\) 0.5, CaCl\(_2\) 2.7, and dextrose 5.5, equilibrated with 95\% O\(_2\)-5\% CO\(_2\). The coronary sinus was opened along its longitudinal axis and carefully dissected away from the adjoining atrial and ventricular muscle, as previously described.7-8 Small strips of coronary sinus (1x3 mm) were secured in a Lucite tissue bath, which was perfused continuously with Tyrode’s solution at a rate of 13 ml/min. This resulted in an exchange of bath volume every 30 seconds. The perfusate was equilibrated continuously with a gas.
mixture of 95% O₂-5% CO₂ to maintain the pH in the bath at 7.35–7.40, as measured by a glass bulb pH electrode. The bath temperature was maintained at 37.0±0.2°C by passing the perfusate through a water-jacketed condenser.8

Preparations of coronary sinus were stimulated with square pulses of 0.75–1.5 msec and amplitudes of 1.5–2 times threshold via bipolar silver wires insulated to the tip with Teflon. Transmembrane potentials were recorded via 3M KCl-filled glass capillary microelectrodes with tip resistances of 20–40 M Ω. The signals were processed through an amplifier with high input impedance and capacity neutralization and displayed on an oscilloscope and strip chart recorder. The equipment was calibrated with a 100 mV sawtooth pulse with a rise time of 200 V/sec as previously described.8,9

After stimulating preparations at a basic cycle length of 600 msec for a 60-minute equilibration period, cycle length was increased to 1,000 msec for 15 minutes, and control measurements of maximum diastolic potential, action potential amplitude, V₃max of phase 0, and duration at 30%, 50%, and 90% repolarization (APD₉₀, APD₅₀, and APD₀, respectively) were made.8,9 Preparations were driven at cycle lengths from 1,000 to 200 msec for 15 beats, and the drive was stopped periodically to observe for delayed afterdepolarizations and triggered activity. When afterdepolarizations occurred, their amplitudes and coupling intervals to the previous action potentials were measured.8,9

**Pharmacological Interventions**

Preparations were exposed to Tyrode’s solution containing graded concentrations of either 1-epinephrine or isoproterenol (1×10⁻⁷–1×10⁻⁵ M) for 10 minutes at each concentration. Perfusates of appropriate concentration were made immediately before use by dilution of stock solutions that were prepared daily. All solutions contained EDTA (5×10⁻⁵ M) to retard oxidation of the catecholamine and were kept in darkly tinted glass bottles to minimize degradation by light. Prior work5,11 as well as preliminary studies performed as part of these experiments (data not shown) demonstrated no effect of this concentration of EDTA on the transmembrane potential and impulse initiation in Purkinje or coronary sinus fibers.

Other preparations were superfused with 2×10⁻⁷ M ouabain alone or in the presence of 1×10⁻⁵ M isoproterenol until delayed afterdepolarizations ≥5 mV in amplitude appeared.1,10 The duration of exposure that was required varied from 18 to 60 minutes with a mean of 38 minutes in the adult and 45 minutes in the young preparations.

**Biochemical Methods**

**Preparation of canine coronary sinus membranes.** Membranes were prepared from individual and pooled specimens of adult coronary sinus; for the young dogs, groups of five to six were pooled. Tissue was weighed, minced, and homogenized (Polytron, speed 8 and 10 seconds, Brinkmann Instruments, Westbury, New York) in a 4-vol excess of 0.25 M sucrose, 0.03 M histidine, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.0. The crude homogenate was centrifuged at 1,500g for 20 minutes. The remaining supernatant was subsequently centrifuged at 40,000g, and the resulting pellet was resuspended in approximately 1 ml buffer (2.5 mg/ml). Protein was determined according to the method of Lowry et al.12 Tissue was stored at −70°C in aliquots and was thawed only once for assays of adenylate cyclase, β-receptors, and G proteins.

**β-Adrenergic receptor binding assay.** β-Adrenergic receptors in membrane preparations were assayed according to standard radioligand binding techniques.13 Canine coronary sinus membrane tissue (50 μg) was incubated with 25 μl [¹²⁵I]iodocyanopindolol at final concentrations of 20–200 pM, for 30 minutes at 37°C in a buffer consisting of NaCl (0.14 M), KCl (0.01 M), dextrose (2 mg/ml), bovine serum albumin (1 mg/ml), and Tris-HCl (0.01 M, pH 7.4). After rapid filtration over Whatman GF/B glass filters (Clifton, New Jersey) and two washes with 5 ml incubation buffer, radioactivity retained by the filters was detected in an autogamma scintillation spectrometer (Packard Instruments, Downers Grove, Illinois). Specific binding was determined by the difference in binding with and without propranolol (10 μM) and was typically >75% of total binding. A Scatchard analysis was performed to determine β-adrenergic receptor density and affinity (Kᵦ and Bₘₐₓ).14 This was done as the average of triplicate binding determinations. Adult canine coronary sinus binding experiments were done, and the mean±SEM for these values is reported (n=3). Young canine coronary sinus tissue was pooled (n=6) to obtain adequate quantities of membrane protein. The Scatchard analysis is based on triplicate determinations.

**Identification and quantification of regulatory proteins.** ADP-ribos e is covalently linked to the alpha subunit of the stimulatory guanine nucleotide regulatory protein (Gₛ) or the inhibitory guanine nucleotide regulatory protein (Gᵢ) in the presence of cholera or pertussis toxin, respectively. Gₛ and Gᵢ were quantified by determining the incorporation of [³²P]ADP-ribose into membrane protein using [³²P]nicotinamide adenine dinucleotide (NAD) as the substrate according to the methods of Kaslow et al15 as previously modified by us.16 Cholera or pertussis toxin was activated by incubation with dithiothreitol (20 mM) for 10 minutes at 37°C. Twenty-five microliters of cardiac membrane (35–50 μg) were incubated in 65 μl buffer containing 50 mM K₃PO₄, 10 units aprotinin, 13 mM thymidine, 3.2 mM ADP-ribose, 13 mM arginine, 0.2 mM GTP (for pertussis) or 0.2 mM nonhydrolyzable analogue of GTP, Gpp(NH)p, (for cholera), 10 μM [³²P]NAD (18–54 Ci/mmol), and 18 μg activated cholera toxin or 2 μg...
activated pertussis toxin. The reaction was terminated by the addition of 1 ml ice-cold 7% trichloroacetic acid (TCA) and centrifugation at 12,000g. The pellet was then suspended in 1% TCA, recentrifuged, and subsequently solubilized with sodium dodecyl sulfate sample buffer. Electrophoresis was performed on slab gels at 300 V for 3 hours. Gels were then stained with Coomassie blue and analyzed by autoradiography using Kodak XRP-5 film. The concentrations of $G_i$ or $G_o$ were calculated from the number of counts in the labeled bands of interest (42 kDa and 39 kDa, respectively) and the specific activity of $^{32P}$NAD. ADP-ribosylation experiments were performed on two different preparations of pooled adult canine coronary sinus. For ADP-ribosylation experiments on young canine coronary sinus membranes from pooled specimens ($n=6$) were used twice.

**Data Analysis**

Electrophysiological data ($X\pm SEM$) are reported only from preparations in which a single microelectrode impalement was maintained throughout the experiment. Statistical comparisons of action potential parameters from the adult and young age groups were derived from Student's $t$ test. Developmental changes in action potential characteristics were evaluated by linear regression analysis of each parameter versus age. The significance of correlations was determined by an $F$ test. Adult and young dose-response curves were compared by analysis of variance nested for age. Biochemical data were compared by Student's $t$ test.

**Table 1. Control Transmembrane Potentials**

<table>
<thead>
<tr>
<th>MDI (mV)</th>
<th>Amp (mV)</th>
<th>$V_{max}$ (V/sec)</th>
<th>APD$_{50}$ (msec)</th>
<th>APD$_{90}$ (msec)</th>
<th>APD$_{99}$ (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult ($n=22$)</td>
<td>-82±1</td>
<td>116±4</td>
<td>268±20</td>
<td>50±3</td>
<td>85±3</td>
</tr>
<tr>
<td>Young ($n=29$)</td>
<td>-79±1</td>
<td>107±1*</td>
<td>168±16*</td>
<td>43±1*</td>
<td>58±2*</td>
</tr>
</tbody>
</table>

Values are mean±SEM for all control experiments on adults and young animals ≤40 days of age. Basic cycle length is 1,000 msec. Subsequent text and figures give values for subsets of these two groups; hence, the control data in various experiments will differ somewhat from the controls in Table 1.

*p<0.05 compared with adult.

**Results**

**Electrophysiological Studies**

Developmental changes in the transmembrane action potential. Action potential parameters at a drive cycle length of 1,000 msec in coronary sinus preparations from 29 dogs ≤40 days of age and from 22 adult dogs are shown in Table 1 and Figure 1. No phase 4 depolarization occurred, and there was no significant difference in maximum diastolic potential between the two groups. However, action potential amplitude, $V_{max}$ of phase 0, and action potential duration were greater in the adult preparations. To document more completely the developmental changes in these variables, an additional 23 preparations from dogs 40–73 days of age were studied. A significant correlation was found between action potential duration and age over the range of 0–73 days. Data for APD$_{90}$ are presented in Figure 2; the other durations measured showed a comparable correlation (data not shown). No such correlation occurred for action potential amplitude and $V_{max}$ of phase 0.

To compare the relative sensitivities of adult and young coronary sinus preparations with $\beta$-agonist, the effects of isoproterenol and of epinephrine on maximum diastolic potential and action potential duration were analyzed. Comparable results were

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** Transmembrane potential recordings from one adult and one young preparation (<40 days of age) in control Tyrode's solution and after a 10-minute exposure to isoproterenol. Basic cycle length is 1,000 msec.
Figure 2. Plot of developmental changes in action potential duration (APD) at 50% repolarization in coronary sinus preparations from dogs aged 0–73 days paced at basic cycle length of 1,000 msec. Each point represents the mean of APD measurements from one to eight impalements in each of 52 preparations from 52 animals. (Regression equation: $y = 0.4x + 50; r = 0.7$, p < 0.05). Comparable data were found for APD$_{90}$ and APD$_{99}$ but are not presented here.

Obtained with both catecholamines, and only data for isoproterenol are presented here. As demonstrated in Figure 3, the dose-response relation of maximum diastolic potential to isoproterenol concentration is superimposable in the two age groups, indicating a similar sensitivity of the adult and young tissues to β-adrenergic stimulation. After exposure to isoproterenol, the action potential plateau was prolonged in both age groups. However, the plateau duration in the young tissue after maximal isoproterenol stimulation never exceeded that of the adults recorded during the control state (Figure 4).

Developmental changes in the induction of delayed afterdepolarizations: Effects of catecholamines. Coronary sinus preparations from eight adult dogs were superfused with epinephrine, $1 \times 10^{-7} - 1 \times 10^{-5} \text{ M}$. After exposure to each concentration, trains of 15 stimuli were delivered at cycle lengths of 1,000, 800, 600, 400, and 200 msec. A typical response is shown in Figure 5 at an epinephrine concentration of $1 \times 10^{-6} \text{ M}$. A delayed afterdepolarization is seen after the last paced beat in the top three panels. As previously described, its amplitude increases with shortening of the pacing cycle length. After stimulation at a cycle length of 200 msec, the delayed afterdepolarization attains threshold, which results in a train of triggered action potentials. The amplitude of the delayed afterdepolarizations also increased with increasing epinephrine concentrations as shown in Figure 6, and 88% of the adult preparations developed triggered activity. The responses of fourteen 0–36-day coronary sinus preparations to the same protocol were in sharp contrast with those of the adults, as shown in Figures 5 and 6. At epinephrine concentrations $\leq 1 \times 10^{-5} \text{ M}$, pacing at all cycle lengths produced neither afterdepolarizations nor triggered activity.

In a parallel series of experiments with isoproterenol $1 \times 10^{-7} - 1 \times 10^{-5} \text{ M}$, preparations from the hearts of 10 additional dogs, 9–37 days of age, showed no delayed afterdepolarizations or triggered activity. However, seven of 10 preparations from slightly older dogs (41–50 days) did develop delayed afterdepolarizations, and three preparations showed triggered activity during the same protocol. In contrast, all nine adult coronary sinus preparations subjected to isoproterenol superfusion and the same pacing protocol developed delayed afterdepolarizations, and six developed triggered activity.
In summary, the generation of delayed afterdepolarizations and triggered activity in response to \( \beta \)-adrenergic catecholamines never occurred before 37 days of age, was seen intermittently between 40 and 50 days of age, and was universally observed in adult tissues.

Developmental changes in the induction of delayed afterdepolarizations: Effects of ouabain. Delayed afterdepolarizations are thought to result from a transient inward current that is dependent on the accumulation of intracellular free calcium ([Ca\(^{2+}\)]. The Na\(^{+}\)-Ca\(^{2+}\) exchanger is also thought to play an important role here (see Reference 19 for review). Because \( \beta \)-agonist-induced delayed afterdepolarizations were never seen in coronary sinus preparations less than approximately 40 days of age, we considered whether other interventions that increase [Ca\(^{2+}\)]\(_{i}\) might induce them. Ouabain increases [Ca\(^{2+}\)]\(_{i}\) by inhibiting the sodium-potassium pump and secondarily reequilibrating the Na\(^{+}\)-Ca\(^{2+}\) exchange and releasing intracellular Ca\(^{2+}\) stores (see References 19 for review). If the neonatal canine heart has the cellular machinery to generate delayed afterdepolarizations and is simply lacking a \( \beta \)-adrenergic mechanism, ouabain would be expected to induce them.

Thus, we superfused preparations from eight dogs, 7–33 days of age, with ouabain, 2x \( \times 10^{-7}\) M. Delayed afterdepolarizations did occur. Trains of 15 stimuli were delivered at cycle lengths from 1,000 to 200 msec with each train followed by a brief quiescent period to allow for the emergence of triggered activity. One or two delayed afterdepolarizations were seen in all eight young preparations, but none developed triggered activity (see Figure 7). Another six preparations were superfused with a combination of ouabain and isoproterenol \( 1 \times 10^{-7}\) M. All developed delayed afterdepolarizations, and one (a 41-day preparation) produced a single triggered beat after a train of 15 stimuli at a 200-msec cycle length. The same protocol was used to study the effects of ouabain alone on six adult preparations. All developed one or two delayed afterdepolarizations although none showed triggered activity. Hence our electrophysiological experiments suggested that both neonatal and adult coronary sinuses are capable of generating delayed afterdepolarizations in response.
FIGURE 7. Tracings showing delayed afterdepolarizations induced by ouabain (2×10⁻⁷ M) in a 24-day coronary sinus. One or two afterdepolarizations follow the last impulse of each 15-beat train. BCL, basic cycle length.

to ouabain but that only the adults manifest this phenomenon in response to β-adrenergic stimulation.

Biochemical Studies of the β-Adrenergic Receptor Complex

The aforementioned results focused our attention on the β-receptor complex itself as a possible site of the fundamental difference between neonatal and adult coronary sinus preparations. Therefore, we analyzed the β-receptor, the guanine nucleotide coupling proteins, and a known biochemical effector of the β-adrenergic response, adenylate cyclase.

The Bₐₐₐ for β-adrenergic receptors was similar in the adult (48±4.8 fmol/mg) and the young (56 fmol/mg) canine coronary sinus membranes. There was also no difference in the Kᵣ of adult and young tissue (60 and 55 pM, respectively). Gᵢ also was similar in amount between adult (6.2 fmol/mg) and young (6.5 fmol/mg) coronary sinus. However, Gᵢ was 2.5-fold greater in the adult (15 fmol/mg) than in the young (6.0 fmol/mg). An example is shown in Figure 8.

Adenylate cyclase activity was measured in response to Gpp(NH)p, isoproterenol plus Gpp(NH)p, and the diterpine, forskolin. Maximal Gpp(NH)p, isoproterenol plus Gpp(NH)p, and forskolin-stimulated adenylate cyclase activities were all greater in the adult than in the young coronary sinus (Table 2). Despite the greater absolute values for stimulated activities in adult membranes, however, the young canine coronary sinus showed a greater-fold increase in stimulated activities over basal values (Table 2, Figure 9). The smaller-fold increase in these three stimulated activities is consistent with a greater influence of Gᵢ in the adult as compared with the young canine coronary sinus.

TABLE 2. Adenylate Cyclase Activity in Young and Adult Canine Coronary Sinus

<table>
<thead>
<tr>
<th></th>
<th>Adult</th>
<th>Young</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>375±10</td>
<td>121±4*</td>
</tr>
<tr>
<td>ISO (0.5 mM)</td>
<td>481±10</td>
<td>200±16*</td>
</tr>
<tr>
<td>Gpp(NH)p (0.1 mM)</td>
<td>1,397±241</td>
<td>1,199±69</td>
</tr>
<tr>
<td>Forskolin (0.5 mM)</td>
<td>4,303±334</td>
<td>2,459±210*</td>
</tr>
<tr>
<td>ISO (0.5 mM)+Gpp(NH)p (0.1 mM)</td>
<td>1,648±65</td>
<td>1,599±18</td>
</tr>
</tbody>
</table>

Data are shown as mean±SEM (pmol/mg/min) for an experiment performed in triplicate. ISO, isoproterenol. *p<0.05

FIGURE 8. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of ADP-ribosylated proteins in young and adult canine coronary sinus tissue. The cholera toxin substrate (CT) is similar in both sinus tissues. However, for pertussis toxin (PT) there is an increase in the amount of ³²P incorporation into the band migrating as Gᵢ in the adult as compared with the young. A crude membrane preparation from an individual young coronary sinus tissue (crude) and membranes from six pooled specimens (pooled) are shown.
Adrenergic sensitive adenylate cyclase activity denoted
Henning and Wit20 have shown that prolongation of
plateau is determined, in part, by the entry of Ca^{2+}
into the cell via the slow inward current. Moreover,
adenergic stimulation prolonged repolarization
at both ages, never did the plateau of the young
equal or exceed the duration of even
coronary sinus may relate to age-dependent length-
ness of all activities mediated by β-adrenergic
catecholamines. For example, changes in maximum
diastolic potential induced by isoproterenol were
depressed end-organ responsiveness (measured as left ventricular dP/dt in
response to catecholamine stimulation).26 However, a putative developmental change in the β-
receptor complex does not dampen the responsiveness of all activities mediated by β-adrenergic
catecholamines. For example, changes in maximum
diastolic potential induced by isoproterenol were
identical in young and adult tissues. Thus, at least in
the context of membrane potential, the young cor-

diagram
Coronary sinus responds competently and completely to β-adrenergic stimulation.

It is unlikely that differences in the β-receptor per se can account for development of β-adrenergic mediated delayed afterdepolarizations in the adult because β-receptor density and affinity were unchanged. Similarly, the stimulatory guanine nucleotide binding protein, Gα, that links the β-receptor to adenylate cyclase activity was the same when young and adult coronary sinus tissue were compared with one another. It is possible that, despite the greater inhibitory properties of Gα in the adult, isoproterenol-stimulated adenylate cyclase activity superimposed on a higher basal adenylate cyclase level is of a sufficient magnitude to increase [Ca2+]i above a threshold required for afterdepolarizations to occur (see Table 2). In this regard, it is of interest to relate the changes in adenylate cyclase activity shown in Table 2 to the effects of isoproterenol on action potential duration (Figure 4). Both basal and maximal isoproterenol-stimulated values for action potential duration and adenylate cyclase activity in the adult exceed those of the young, a result consistent with our studies of canine ventricular myocardium.27 In addition, at maximal stimulation, the adenylate cyclase activity and action potential duration for the young are equal to or less than the corresponding adult values. Adenylate cyclase activity, which augments Ca2+ entry, may provide a mechanism for the developmental differences in response to β-adrenergic stimulation. Moreover, this pathway is distinct from the mechanisms whereby ouabain stimulation leads to elevated intracellular Ca2+. However, this argument assumes that the absolute level of adenylate cyclase activity (higher in adults) is more important with regard to accumulation of [Ca2+]i than is the relative change induced in adenylate cyclase activity by β-agonist (higher in the young).

The observed increase in Gα is of interest in its own right. If the pertussis toxin substrate is linked to β-adrenergic stimulation of adenylate cyclase activity, adult coronary sinus, enriched in Gα, should show blunted adrenergic stimulation of adenylate cyclase activity in comparison with young tissue. Indeed, isoproterenol-stimulated adenylate cyclase activity in adult coronary sinus is reduced when expressed as a percentage of maximal stimulatable activity. These observations suggest that Gα is an important component in the actions of β-adrenergic catecholamines that influence adenylate cyclase activity in coronary sinus. Previous studies have also shown that Gα is influential in accounting for expression of β-agonist stimulated adenylate cyclase activity.28

In conclusion, we have found that the developmental change in catecholamine-induced delayed afterdepolarizations and triggered activity in canine coronary sinus appears to be related importantly to developmental changes in the duration of the action potential. That the young tissues have the cellular “machinery” to induce afterdepolarizations is clearly seen in the ouabain experiment. Moreover, the β-receptor-effector complex appears comparable at both ages with the exception of Gα, to which a role in development of triggered activity is not readily attributable. With this in mind, the change in action potential duration appears to be the most likely factor in the genesis of triggered arrhythmias in coronary sinus.

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References

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