Ontogeny of humoral heart rate regulation in the embryonic mouse

GEORGE A. PORTER, JR. AND SCOTT A. RIVKees
Department of Pediatrics, Division of 1Cardiology and 2Endocrinology,
Yale University School of Medicine, New Haven, Connecticut 06520
Received 2 November 2000; accepted in final form 15 March 2001

Porter, George A., Jr. and Scott A. Rivkees. Ontogeny of humoral heart rate regulation in the embryonic mouse. Am J Physiol Regulatory Integrative Comp Physiol 281: R401–R407, 2001.—Catecholamines, acetylcholine, and adenosine are known to influence cardiac function, yet the effects of these agents on mammalian embryonic myocardium are largely unknown. To address this issue, we compared the chronotrophic effects of adenosinergic, adrenergic, and muscarinic agents on cultured murine embryos from postcoital day (PC) 8.0, when the fusing heart tubes first begin to beat, to PC 14, when cardiogenesis is essentially complete. At PC 8.0 and older, A1-adenosine receptor (A1AR) activation significantly decreased heart rates. Adrenergic stimulation caused modest increases in heart rates (145–155% of baseline) beginning at PC 9.0. Muscarinic activation decreased heart rates only after PC 13. When receptor gene expression was examined, A1ARs and β2ARs were expressed in isolated hearts as early as PC 9.0, and β2ARs and m2-muscarinic receptor genes were expressed at PC 11.0. These results identify the adenosinergic system as the earliest and most potent regulator of embryonic cardiac function and show that prenatal responsiveness to catecholamines and acetylcholine develops at later embryonic stages.

In mature and developing mammals, cardiac output is influenced by heart rate, stroke volume, and contractility (1, 4, 5, 19, 30). However, in embryos, available evidence suggests that heart rate plays a particularly important role in regulation of cardiac output (1, 4, 5). Factors that alter heart rate may greatly influence cardiac output at early developmental stages.

Previous work has shown that A1-adenosine receptors (A1ARs) are among the earliest expressed G protein-coupled receptors in the heart, with expression first observed at postcoital day (PC) 8.0 in rat embryos (22). A1AR activation potently slows embryonic heart rates as early as PC 9.5, the earliest age examined (9). Theophylline, an adenosine receptor antagonist, increases embryonic mouse heart rates as early as PC 12 (31).

Adrenergic receptors have been detected in mouse hearts at PC 13 and in the rat at PC 12 (2, 25, 29). Increases in heart rates due to adrenergic stimulation have been observed as early as PC 9.5 in mice and at PC 10.5 in rats (8, 15, 23).

Muscarinic receptor mRNA has been detected in rat cardiac tissue at PC 18 (6), and receptor binding sites are present at PC 15 in the mouse (18, 25). Muscarinic stimulation alters heart rates at PC 12–13 in mice and at PC 11.5 in rats (8, 23, 25, 31).

Although available evidence shows that embryonic hearts can respond to extrinsic stimulation, we do not know when cardiac responsiveness to adenosinergic, adrenergic, and muscarinic agents first develops nor do we know the relative importance of these factors in regulating embryonic cardiac function. To further define the ontogeny of adenosinergic, adrenergic, and muscarinic influences on embryonic cardiac physiology, we examined the expression and influence of these receptor systems on embryonic heart rates from the inception of spontaneous contractility at PC 8.0 to the end of cardiac organogenesis at PC 14.0.

MATERIALS AND METHODS

Animals. The Yale Animal Care and Use Committee approved all studies. C57J/BL mice were exposed to a 12:12-h dark-light cycle with free access to food and water. Males and females were paired and separated when a vaginal plug was observed. The morning a mating plug was observed was designated as PC 0.5.

Cultures. Dams were anesthetized with carbon dioxide and euthanized by cervical dislocation. To obtain embryos, hysterectomy was performed under sterile conditions, and the uteri were rinsed in Dulbecco's PBS with 2 mM MgCl2 at room temperature. Uteri were then transferred at 37°C into DMEM with 10% fetal bovine serum (Fetal Clone II, Hyclone Laboratories, Logan, UT) and 50 mM HEPES buffer, which was used for all subsequent incubations and studies.

While visualized using a dissecting microscope (Zeiss), embryos were separated from uteri and transferred to fresh media for further dissection. Embryonic age was determined by morphology and somite number (12). Embryos younger than PC 10.0 were incubated intact. For older embryos, isolated hearts were studied, as this is required to maintain a consistent heart rate. Embryos or embryonic hearts were transferred to individual wells with 3 ml of media and incubated at 37°C in a 5% CO2-room air incubator. Dose-response
Hearts rate measurements were performed using visualization with a dissecting microscope. Temperature was maintained between 35 and 38°C using a heated stage. Individual plates containing 4–12 embryos were removed from the incubator and placed on the stage for 5 min. The baseline heart rate of each embryo was measured, and drugs or vehicle was added to each well. Heart rates were determined by direct visual counting for 15 s; two measurements were recorded per sample.

To assess the effects of receptor activation on heart rates, receptor agonists, antagonists, and reuptake blockers were applied to the culture media. Except when noted, the doses ranged from 1 nM to 10 μM. For studies of AR activation, the agonist N6-cyclopentyladenosine (CPA) and the antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) were used. Dipyridamole was used to block the reuptake of endogenous adenosine. Adrenergic receptors were stimulated with isoproterenol and inhibited with alpenrol. Tyramine was used to increase levels of endogenous catecholamines. Bethanecol was used to stimulate muscarinic receptors at doses from 100 nM to 10 mM. Atropine was used to antagonize muscarinic receptors, and neostigmine to inhibit acetylcholinesterase and increase endogenous levels of acetylcholine.

To generate dose-response curves, individual embryos were treated with increasing doses of drug without changing the media. Stock solutions of each drug were made fresh daily (isoproterenol, neostigmine) or kept frozen at 20°C (not shown). Drug and chemicals. PBS, DMEM, and HEPES were obtained from Life Technologies. Adenosine deaminase (ADA) was purchased from Boehringer Manheim (Indianapolis, IN). All other drugs and chemicals were obtained from RBI/Sigma (Natick, MA).

RESULTS

Heart rates. To assess heart rate responses to different pharmacologic agents, we cultured mouse embryos at different stages of cardiogenesis. Before any drugs were applied, baseline heart rates were obtained 1–4 h after dissection, and the length of this preincubation did not appear to affect heart rates. Heart rates ranged from 62.5 beats/min at PC 8.0–8.5 to 119 beats/min at PC 10.0–11.0 (Fig. 1). Heart rates at PC 10.0–12.0 were similar to those reported for mouse embryos studied in vivo using fetal Doppler ultrasonography or in situ via hysterotomy (7, 13, 16, 30). In control experiments, heart rates were found to be stable throughout the time required to perform a dose-response curve, as demonstrated by the lack of significant variation of vehicle-treated specimens (Fig. 2, Table 1).
significantly different from vehicle (P(DPCPX), dipyridamole, isoproterenol, alprenolol, tyramine, atropine, and neostigmine and were 10 mM for bethanecol. *Values are mean heart rate response to each drug as a percentage of baseline ± SE. In all cases, the maximal response to each drug occurred at the highest 2 doses used. Maximal doses were 10 μM for N°-cyclopentyladenosine (CPA), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), didympidomole, isoproterenol, alprenolol, tyramine, atropine, and neostigmine and were 10 mM for bethanecol. *Values are significantly different from vehicle (P < 0.05).

### Responses to adenosinergic agents

Our previous studies demonstrated that A1ARs are expressed in the heart at the inception of rhythmic cardiac contractions and that activation of these receptors slows heart rates as early as PC 9.5 (9, 22). To extend these functional studies, embryonic heart rate responses to CPA (A1AR agonist), DPCPX (A1AR antagonist), and dipyridamole (adenosine reuptake inhibitor) were tested. Embryos were studied from PC 8.0 to PC 14.0. During initial experiments, the Emax to CPA increased markedly between PC 8.0 and PC 10.0. Therefore, specimens were divided into age groups by half-day increments (PC 8.0–8.5, PC 8.5–9.0, PC 9.0–9.5, and PC 9.5–10.0). Older specimens were divided into full-day age groups (PC 10.0–11.0, PC 11.0–12.0, PC 12.0–13.0, PC 13.0–14.0).

Treatment of embryos with 1 nM to 10 μM CPA decreased heart rates in a dose-dependent manner at all ages. However, Emax to CPA treatment increased with age. At PC 8.0–8.5, CPA decreased heart rates to 65.2% of baseline (Fig. 2, Table 1). Over the next 2 days, dose-dependent responses increased until complete asystole was observed after PC 11.0 (Fig. 3, Table 1). At all ages tested, the EC50 was between 9 and 50 nM.

We next studied if adenosine released into the culture medium by the embryo affects heart rates. First, some specimens were treated with ADA, which degrades endogenous adenosine. The baseline heart rates were not different in the presence or absence of ADA (not shown). Second, we treated specimens at all ages with DPCPX. We found no consistent response, although a significant increase in heart rates was observed at PC 12.0–13.0 (Fig. 4, Table 1). Third, we

### Table 1. Maximal responses to each agent at each postcoital age

<table>
<thead>
<tr>
<th>Gestational Age</th>
<th>Vehicle</th>
<th>CPA</th>
<th>DPCPX</th>
<th>Dipyridamole</th>
<th>Isoproterenol</th>
<th>Alprenolol</th>
<th>Tyramine</th>
<th>Bethanecol</th>
<th>Atropine</th>
<th>Neostigmine</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0–8.5</td>
<td>106.5 ± 4.8</td>
<td>65.2 ± 2.8*</td>
<td>108.0 ± 0.9</td>
<td>95.7 ± 11.4</td>
<td>121.4 ± 4.1</td>
<td>103.0 ± 4.5</td>
<td>102.3 ± 3.2</td>
<td>107.3 ± 7.1</td>
<td>117.7 ± 4.2</td>
<td>99.7 ± 10.6</td>
</tr>
<tr>
<td>8.5–9.0</td>
<td>98.2 ± 3.9</td>
<td>24.8 ± 7.1*</td>
<td>110.0 ± 0.9</td>
<td>76.0 ± 5.2</td>
<td>114.5 ± 8.6</td>
<td>98.0 ± 11.0</td>
<td>99.3 ± 5.4</td>
<td>96.2 ± 5.7</td>
<td>96.0 ± 3.8</td>
<td>100.7 ± 4.3</td>
</tr>
<tr>
<td>9.0–9.5</td>
<td>89.3 ± 4.9</td>
<td>20.2 ± 6.9a</td>
<td>94.0 ± 13.0</td>
<td>39.0 ± 22.7e</td>
<td>145.6 ± 15.1e</td>
<td>89.5 ± 6.1</td>
<td>98.5 ± 11.2</td>
<td>108.9 ± 7.3</td>
<td>70.7 ± 4.3</td>
<td>100.7 ± 4.3</td>
</tr>
<tr>
<td>9.5–10.0</td>
<td>98.9 ± 3.6</td>
<td>9.0 ± 6.1a</td>
<td>118.3 ± 14.1</td>
<td>68.8 ± 24.1</td>
<td>154.8 ± 10.3*</td>
<td>86.0 ± 6.8</td>
<td>84.7 ± 5.2</td>
<td>98.3 ± 7.8</td>
<td>89.5 ± 17.4</td>
<td>82.7 ± 1.5</td>
</tr>
<tr>
<td>10.0–11.0</td>
<td>99.0 ± 5.4</td>
<td>16.8 ± 6.8a</td>
<td>108.3 ± 6.6</td>
<td>47.8 ± 15.7a</td>
<td>114.7 ± 6.2</td>
<td>91.3 ± 8.7</td>
<td>83.8 ± 9.6</td>
<td>74.2 ± 7.2</td>
<td>97.0 ± 3.5</td>
<td>114.7 ± 3.9</td>
</tr>
<tr>
<td>11.0–12.0</td>
<td>94.8 ± 8.6</td>
<td>0.8 ± 0.5a</td>
<td>123.0 ± 20.2</td>
<td>60.2 ± 11.8</td>
<td>147.0 ± 11.5*</td>
<td>107 ± 12.1</td>
<td>102.0 ± 15.0</td>
<td>77.2 ± 6.8</td>
<td>116.8 ± 12.7</td>
<td>78.2 ± 9.5</td>
</tr>
<tr>
<td>12.0–13.0</td>
<td>99.8 ± 7.8</td>
<td>0 ± 0a</td>
<td>150.7 ± 20.3</td>
<td>20.5 ± 10.8a</td>
<td>150.3 ± 9.7*</td>
<td>79 ± 4.9</td>
<td>103.3 ± 18.8</td>
<td>76.8 ± 11.5</td>
<td>96.3 ± 9.8</td>
<td>105.3 ± 11.3</td>
</tr>
<tr>
<td>13.0–14.0</td>
<td>98.6 ± 7.7</td>
<td>0 ± 0a</td>
<td>116.0 ± 28.2</td>
<td>16.3 ± 12.1a</td>
<td>149.6 ± 11.9*</td>
<td>102.8 ± 9.8</td>
<td>93.0 ± 11.6</td>
<td>31.6 ± 13.3a</td>
<td>84.7 ± 4.7</td>
<td>106.3 ± 18.6</td>
</tr>
</tbody>
</table>

Values are mean heart rate response to each drug as a percentage of baseline ± SE. In all cases, the maximal response to each drug occurred at the highest 2 doses used. Maximal doses were 10 μM for N°-cyclopentyladenosine (CPA), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), dipyridamole, isoproterenol, alprenolol, tyramine, atropine, and neostigmine and were 10 mM for bethanecol. *Values are significantly different from vehicle (P < 0.05).
treated embryos with dipyridamole, which decreased heart rates at all ages except PC 8.0–8.5 (Fig. 4, Table 1). Thus basal concentrations of endogenous adenosine do not appear to affect embryonic heart rates. Yet, increasing the local levels of adenosine with reuptake inhibitors can decrease heart rates.

Responses to adrenergic and muscarinic agents. We then determined the effects of adrenergic and muscarinic receptor stimulation on heart rate. To assess the ontogeny of adrenergic responsiveness, we treated embryos with the nonspecific β-adrenergic agonist isoproterenol. From PC 8.0 to 9.0, no significant alterations in heart rates were observed, even with maximal doses of isoproterenol (Fig. 5, Table 1). From PC 9.0–10.0 and 11.0–14.0, isoproterenol treatment increased heart rates to E_{max} of 145–155% of baseline (Fig. 5, Table 1) with an EC_{50} of 10–50 nM. Interestingly, from PC 10.0 to 11.0 no significant changes in heart rates were observed in 10 embryos from five different experiments.

We next treated embryos with the β-adrenergic antagonist aloprenolol to determine if endogenous catecholamines alter heart rates in our culture system. Aloprenolol had no significant effect on embryonic heart rates at any age tested (Fig. 5, Table 1). Treatment with tyramine, which increases endogenous catecholamine levels (11), also had no effect at any age (Fig. 5, Table 1). These observations suggest that endogenous catecholamines do not exert significant effects on heart rates during the embryonic period.

Finally, we examined the ontogeny of muscarinic responsiveness using a muscarinic agonist (bethanechol), a muscarinic antagonist (atropine), and an acetylcholine esterase inhibitor (neostigmine). Treatment with bethanechol caused significant decreases in heart rates only at PC 13.0–14.0 (Fig. 6, Table 1). Neither atropine nor neostigmine caused significant alterations in heart rates at any age (Fig. 6, Table 1). Therefore, muscarinic receptor activation appears to have little affect on murine heart rate until late in cardiac development.

Receptor expression assays. Our physiological measurements of receptor activation demonstrated that adenosinergic, adrenergic, and muscarinic receptors become functional at PC 8.0, 9.0, and 13.0, respectively. To assess if receptor expression correlated with these functional data, gene expression was qualitatively examined with PCR. RNA was obtained from specimens at the ages when changes in the physiological responsiveness were observed in the experiments above. In addition, expression in older embryos (PC 17) and neonatal atria and ventricles was performed for
comparison. Using primers specific for mouse A1AR, β1AR, β2AR, and M2MR, we observed that each receptor subtype was expressed in whole murine embryos at PC 8.0 (Fig. 7). Because of their small size, it was not possible to isolate hearts at this age. However, for PC 9.0 and older embryos, we examined RNA obtained from isolated hearts. These experiments demonstrated that A1ARs were expressed from PC 9.0 and later. β1AR was present in very low levels at PC 9.0, and at higher levels in older embryos. β2ARs were expressed in low levels at PC 11.0 and later. M2MR message was detectable at PC 11.0 and at later ages.

**DISCUSSION**

It is generally believed that humoral heart rate control is dependent on the stimulation of adenosinergic, adrenergic, and muscarinic receptors (19, 28). However, the role of these receptor systems play during early development is not known. Our results identify the adenosinergic system as the earliest expressed and most potent humoral regulator of embryonic cardiac function. In addition, we demonstrate that adrenergic and muscarinic receptor activation influences heart rate later in gestation.

At all ages tested, A1ARs were functional. At older ages, A1AR activation more potently influenced heart rates than at younger ages, with a decline in heart rate to 65.2% of baseline at PC 8.0–8.5 followed by a steadily decreasing heart rate until PC 11.0–12.0, when asystole occurred. These data agree with and extend previous observations (9) and demonstrate that heart rates can be regulated from the inception of spontaneous cardiac contractions. These functional data also agree with studies of receptor expression showing A1AR gene expression in isolated hearts as early as PC 9.0. We also found that A1ARs are expressed in whole embryos at PC 8.0. In agreement with our observations, previous studies using in situ hybridization demonstrated that A1ARs are expressed in rat hearts at a developmental stage equivalent to mouse PC 8.5 (22).

Currently, the cellular mechanisms for the change in responsiveness to A1AR activation over time are not known. Our RT-PCR data suggest that increases in A1AR expression may explain some of the increase in responsiveness, but these experiments were not designed to obtain absolute concentrations of A1AR message. Additional developmental changes in the functional coupling of adenosine receptors to their secondary messengers, secondary messenger systems themselves (e.g., G proteins, adenylyl cyclase, or protein kinases), or effector systems (e.g., ion channels or transporters) may also be responsible.

Responsiveness to adrenergic stimulation has been previously demonstrated in the mouse heart as early as PC 9.5 (15). Our data extend the earliest known age of adrenergic receptor expression and function to PC 9.0. Adrenergic control of heart rate from PC 9.0 to 11.0 is most likely due to activation of β1AR, as no β2AR mRNA was detected before PC 11.0. Although our RT-PCR data were not quantified, the suggestion of lower levels of β2AR expression compared with that of β1AR is also consistent with studies in mature mammals (24).

Muscarinic receptor activation decreased heart rates only after PC 13.0, although M2MR gene expression was detected in hearts as early as PC 11.0. It appears unlikely that the discrepancy between receptor expression and functionality would be due to insensitivity in our measurements of heart rates. Therefore, these data suggest that the coupling of these receptors to their secondary messenger systems is also developmentally regulated, as indicated in previous studies (17). Alternatively, before PC 13, cardiac muscarinic receptors might perform functions other than heart rate control.

To determine if endogenous adenosine, catecholamines, and acetylcholine activate their respective receptors during early development, we also tested the effects of receptor antagonists and of agents that increase levels of these endogenous compounds. We saw no consistent alterations in heart rates due to treatment with any antagonist, indicating that local adenosine, catecholamines, and acetylcholine have no effect on basal heart rates under the conditions used. However, profound declines in heart rates were seen in all but the youngest embryos (PC 8.0–8.5) after treatment with dipyridamole. Therefore, although basal levels of intracellular adenosine have little effect on heart rates under these conditions, increases in the concentration of endogenous adenosine have dramatic effects. In contrast to adenosine reuptake studies, the absence of response to treatment with tyramine and neostigmine.

**Fig. 7.** RNA expression of A1-adenosine receptor (A1AR), β-adrnergic receptor (AR), β2AR, and M2 muscarinic receptor (M2MR). RT-PCR was performed using RNA from whole embryos at PC 8.0 (lane 1); from embryonic hearts at PC 9.0 (lane 2), 11.0 (lane 3), 13.0 (lane 4), and 17.0 (lane 5); or from neonatal atria (lane 6) or ventricles (lane 7). All receptors are expressed in whole embryos at PC 9.0, but only A1AR and low levels of β1AR are present in PC 9.0 hearts (as demonstrated by increased exposure of lane 2). All receptors are expressed at PC 11.0 and later, but at different relative concentrations. RT-PCR of 18s rRNA in each specimen is presented to compare relative levels of RNA between samples.
suggests that there are insufficient stores of catecholamines and acetylcholine present to alter heart rates at these stages of development.

We recognize some limitations of our approach. The embryonic culture system used allowed us to reliably assess changes in heart rates but was not an in vivo model. From PC 8.0 to 10.0, embryos were also cultured without an intact placenta, and from PC 10.0 to 14.0, cultures of isolated hearts were studied to maintain rhythmic beating. Under these conditions, the preload and afterload placed on the embryonic heart are nonphysiological. In addition, these isolated hearts lack any sympathetic or parasympathetic input. Although the effects of these conditions on heart rate control in the developing embryo are unknown, there is evidence that such conditions did not significantly affect our results. First, we observed that baseline heart rates using this culture system were very similar to those obtained in vivo in mice (7, 13, 16, 30). Second, although heart rate is dependent on stroke volume and thus preload, afterload, and contractility via neural feedback loops in mature mammals, the sympathetic and parasympathetic neurons first innervate the heart much later in gestation than the ages we studied (6, 20). Furthermore, we are unaware of other systems in which one may treat individual murine embryos with specific concentrations of drugs for a prolonged period without either affecting maternal or placental physiology.

In summary, we demonstrate that A1ARs influence heart rates beginning at PC 8.0, adrenergic receptors affect heart rates after PC 9.0, and muscarinic receptors affect heart rates only after PC 13. We also report that adenosine receptor activation has a more profound effect on embryonic heart rates than that of either adrenergic or muscarinic receptor activation. These studies identify the adenosinergic system as the most potent humoral regulator of mammalian embryonic cardiac function.

Perspectives

These observations emphasize the importance of local control of heart rate in the early embryo. The maintenance of cardiac output is vital to embryonic growth and survival (13, 26, 30). In chicken and mouse embryos, cardiac output appears to be largely dependent on heart rate (4, 5, 13, 30). Thus local, adenosine-mediated changes in heart rates would be expected to have a more profound effect on cardiac output than activation of adrenergic or muscarinic receptors, which depends on external sources of agonist (6, 20, but see Ref. 10).

In comparison with catecholamines and acetylcholine, adenosine also is an ideal regulator of embryonic cardiac function. Unlike catecholamines and acetylcholine, intracellular adenosine concentrations are not dependent on intercellular stores. All cells constantly produce this nucleoside, and conditions that favor the breakdown of ATP, such as hypoxia or increased cellular metabolism, lead to an increase in extracellular adenosine concentrations. It is well recognized in large mammals that fetal heart rates fall during hypoxic stress (21), when levels of both adenosine and catecholamines rise (3, 14, 27). Similar effects might be expected in small mammals, but we are unaware of such experiments. Our observations in cultured embryos, though, indicate that this phenomenon reflects the responsiveness of heart rate to adenosine and show the dominance of adenosine action in the embryonic period. These data may also explain the mechanism of stress-induced fetal bradycardia.

G. A. Porter, Jr., is a Pfizer Postdoctoral Fellow. This work was supported by National Heart, Lung, and Blood Institute Grant HL-58442 to S. Rivkees. S. Rivkees is a Donaghue Medical Research Foundation Investigator.

REFERENCES


