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Autonomic Receptors of the Early Rat Embryo Heart: Growth and Development[1] (39310)

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(Introduced by W. G. Guntheroth)

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The adult heart rate responds to stimulation of the sympathetic and parasympathetic nervous system. These responses are mediated by the release of norepinephrine and acetylcholine (ACh) which act on beta-adrenergic and on muscarinic-cholinergic receptors, respectively. During rat embryogenesis, the heart commences to beat during Day 10 and circulation is established before these nerves reach and innervate the heart on Day 15 (1-3). The effect of ACh on the beat rate of excised rat embryo hearts has been reported by two groups of workers and their conclusions differed; Hall (4) observed a decrease in rate in the Day 11 1/2 and older embryos but Pager *et al.* (5) could not demonstrate this effect until Day 16 1/2. Our studies using the whole explanted embryo suggest an explanation for this discrepancy.

Cholinergic receptors can be experimentally distinguished into two types by (a) their response to atropine, which selectively blocks muscarinic receptors, and (b) to curare, which selectively blocks nicotinic receptors. The experiments reported in this paper demonstrate the presence of cholinergic receptors in the preinnervated intact embryonic heart, consistent with Hall's result in the excised heart, classify them as being of the muscarinic type and show that they are independent of the adrenergic receptors in their action and time of functional appearance. We have found (6) in the explanted whole embryo that the adrenergic response of the heart appears during Day 10 concommitant with the onset of heart function, a result similar to that of Hall's with excised hearts. Our findings indicate that cholinergic response first appears approximately 1/2 day later in development than the adrenergic response, a finding also first observed by Hall.

Methods. Embryos were explanted at either Day 10 1/2 (10–15 somites) or Day 11 (21–26 somites) of gestation. The explantation procedure followed was as described in New (7), Shepard *et al.* (8), and Robkin *et al.* (9). Before each experiment was begun, the explanted embryos in circulating serum were carefully examined with a dissecting microscope. The circulation in the yolk sac was determined to be vigorous without areas of sludging, which is associated with damaged embryos that do not do well in culture. Before drugs were administered, the heart rates were recorded for about 10 min. Only those embryos whose heart beats were strong, with rates greater than 100 bpm and constant were used for the experiments.

The heart rates of the embryos were monitored by a technique of transillumination whereby an image of the heart is projected onto the face of a photomultiplying tube and the heart beat is recorded electronically onto a strip chart recorder (7). The accuracy of rate determination was about 1%. The somite number was determined visually within 1 hr of the termination of the experiment.

The drugs which were studied were carbamylcholine (Carbachol, Merck and Co.), a nonmetabolizable analog of acetylcholine; curare (*d*-tubocurarine chloride, Matheson, Coleman and Bell Chemical Co.); and isoproterenol (Isuprel, Winthrop Laboratories), a specific agonist of the beta-adrenergic receptor. In addition, a few preparations were tested with propranolol (Ayerst Labo-

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ratories) to demonstrate the beta-adrenergic blockage and two preparations were tested with nicotine (nicotine bitartarate, City Chemical Co.) to test for the presence of nicotinic receptors. The threshold for carbamylcholine effect was determined for these experiments to be approximately 20–30 μg/ml of culture medium. Preliminary experiments with atropine blockage following carbamylcholine-induced bradycardia gave variable results below an atropine concentration of 70 ng/ml. We interpreted this concentration to be in the threshold range but did not actually determine the atropine threshold for the embryos.

In order to have strong agonist responses to these drugs and positive blockage, the circulating concentrations used were approximately: carbamylcholine, 100 μg/ml; atropine, 150 ng/ml; tubocurarine, 300 ng/ml; isoproterenol, 400 ng/ml; and propranolol, 500 ng/ml. The curare concentration was chosen arbitrarily to have the same molarity as the atropine which has a strong effect at this concentration. The isoproterenol and propranolol dosages were as used in previous experiments (6).

Drugs were diluted in Hank's balanced salt solution so that 0.1 ml of solution contained the entire dose. Circulators with approximately 10 ml of circulating medium were used in each day's experiment.

The drugs were administered in the order carbachol, curare, atropine, isoproterenol, and when it was used, propranolol. The heart beats of the embryos were followed for 10 to 15 min after each drug administration. Observations (6) have shown that the embryos respond to the agonists and antagonists within 2 min of administration of the drugs. Generally about 10 min is sufficient for the heart rate to stabilize at a new rate.

The responses of the sets of embryos to the various drugs were compared using the Student's t test (10, 11). In addition, the 14- and 15-somite embryos were deleted from the Day $10^1/_2$ set and compared to the Day 11 and the residual Day $10^1/_2$ sets as individual samples from normal distributions. The figure of merit used in the latter case was the probability of exceeding by chance the observed difference from the mean measured in standard deviation units. This comparison was motivated in retrospect by the observation that these two embryos displayed intermediate responses.

In addition, sequential doses from 0.03 to 300 μg/ml of nicotine bitartarate were applied in two separate experiments to Day 11 embryos.

Results. Ten embryos from two litters at Day $10^1/_2$ and 10 embryos from three litters at Day 11 were successfully cultured, treated, and recorded. A summary of the responses to the various drugs is shown in Table I. The response of the 14- and 15-somite embryos of the Day $10^1/_2$ set were sufficiently different from the rest of their cohort that the $10^1/_2$ day results are given both with and without these two in the data set.

Figures 1 and 2 show representative responses of a Day $10^1/_2$ (10 somite) and a Day 11 (26 somite) embryo. The data points shown are as recorded and were read from the strip chart with approximately ± 1 bpm uncertainty. This reading uncertainty is trivial compared to the normal biological variation between embryos and has been generally ignored in the data analysis.

The Day $10^1/_2$ embryos had a significantly lower heart rate than did the Day 11 embryos ($P < 10^{-6}$; see Table I). In addition, the 15-somite embryo lay clearly between the two sets with significant differences between its heart rate and the mean rates for Day 11 ($P < 0.02$) and Day $10^1/_2$ ($P < 0.006$). The 14-somite embryo had a heart rate which was significantly different from the Day 11 set ($P < 0.006$) but not from the Day $10^1/_2$ set ($P < 0.1$).

There was a clear and significant difference in the response to carbamylcholine between the two sets ($P < 10^{-4}$). The Day 11 embryos responded by deceleration of the heart rate by 15% while the Day $10^1/_2$ embryos dropped only 2.5%. In addition, the 14- and 15-somite embryos had responses to carbamylcholine which were not different from the Day 11 set ($P < 0.1$), but were clearly different from the Day $10^1/_2$ set ($P < 10^{-4}$). Average response of the remaining Day $10^1/_2$ embryos to carbamylcholine were not significantly different from zero ($P < 0.09$).

The mean heart rate response of the Day 11 group to curare following the carbamylcholine was a decrease which was different

TABLE I. PERCENTAGE CHANGE IN HEART RATE FROM EQUILIBRIUM RATE AFTER PREVIOUS DRUG.

		Baseline (bpm)	Carbamyl-choline (%)	Tubocura-rine (%)	Atropine (%)	Isoprote-renol (%)
Day 11 embryos, n = 10	mean	175	−15.3	−2.1	15.9	13.3
(21–25 somites)	SE	5.1	2.1	0.8	3.4	2.0
Day 10½ embryos, n = 10	Mean	130	−2.5	0.55	1.8	12.7
(10–15 somites)	SE	2.1	1.0	0.4	0.5	0.8
Day 10½ embryos, n = 8	Mean	128	−1.2	0.20	1.8	12.2
(10–13 somites)	SE	1.9	0.6	0.5	0.6	1.0
15-somite embryo	Observed value	142	−7.0	1.5	1.5	14.7
14-somite embryo	Observed value	135	−8.9	2.4	2.4	14.7

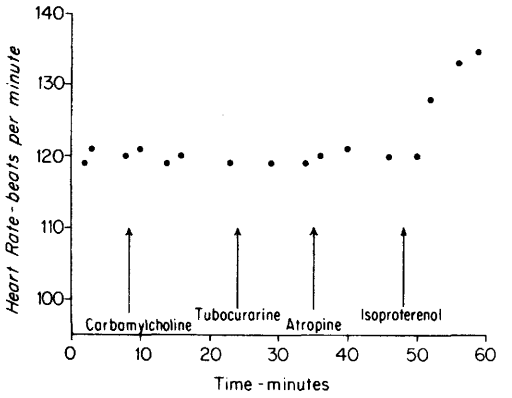

FIG. 1. Heart rate response of 10-somite rat embryo to the sequential administration of drugs. Concentration of drugs added to the circulating medium were: carbamylcholine, 100 μg/ml; atropine, 150 ng/ml; tubocurarine, 300 ng/ml; isoproterenol, 400 ng/ml.

from zero with only marginal significance ($P < 0.04$), while the Day 10½ group was unresponsive ($P < 0.25$).

Both the Day 11 and the Day 10½ groups had clear acceleration following the atropine, $P < 0.001$ and $P < 0.004$, respectively (each compared to zero response). However, the response of the reduced Day 10½ group was less significant ($P < 0.02$). The responses are also significantly different from each other ($P < 0.003$) although the responses of the Day 10½ embryos were of small magnitude compared to the Day 11 set. A scatter plot comparison between the two sets of embryos is shown in Figure 3.

No response to nicotine was found using 0.03, 3.0, and 30 μg/ml of medium. A strong decrease in the heart rate was observed at 300 μg/ml. This dose did not kill the embryos during a 2-hr period of observation; their heart rates merely readjusted to lower values.

Discussion. The overall conclusion to which these results lead is that there is a distinct separation in time between the development of the beta-adrenergic receptor of the sympathetic system and the cholinergic receptor of the parasympathetic system. Cholinergic receptor blocking drugs have no effect on the responsiveness of the adrenergic receptor. These receptors seem to have independent action and follow independent timetables for differentation and development.

Also evident is the relatively higher concentration of carbamylcholine compared to isoproterenol required to elicit a threshold and a maximal response. This may be a reflection of the number of receptors present and/or the affinity of such receptors for the appropriate agonist. Extensive studies would be required to resolve this matter.

The observations of the differences in heart rate responses imply that a significant transition is occurring at about 15 somites. It might be noted that this is approximately the end of the stage of development at which the embryo is changing from ventriflexed to dorsiflexed with a subsequent relocation of the heart from almost direct apposition to the yolk sac surface to a more central place within the embryo.

We interpret the apparent slight response to curare of the Day 11 embryo as an artifact related possibly to a continuing small response to the previously administered carbamylcholine. Our conclusion is that these embryos do not respond to *d*-tubocurarine, so that the cholinergic receptor is not of the nicotinic type. In any event, if the response was of the nicotinic type, curare following carbamylcholine would be expected to cause an increase rather than a decrease in the heart rate.

The atropine response identifies the receptor as of the muscarinic type. The lack of

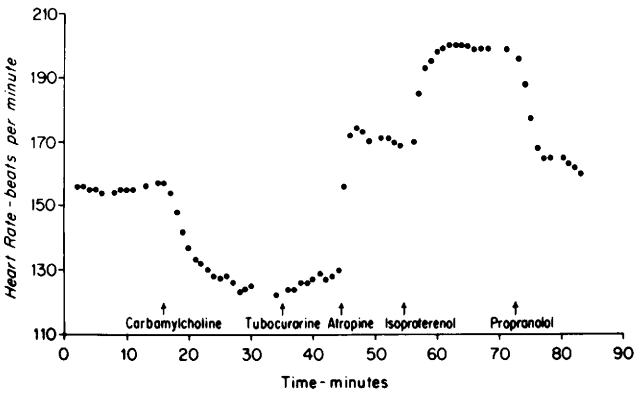

FIG. 2. Heart rate response of 26-somite rat embryo to the sequential administration of drugs. Concentration of drugs in the circulator were as in Fig. 1. The propranolol concentration was 500 ng/ml.

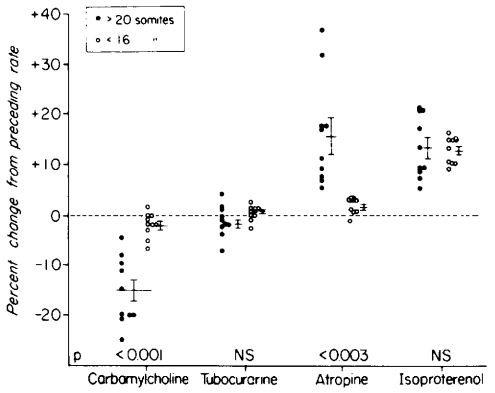

FIG. 3. Scatter plot of the heart rate responses of Day $10^1/_2$ and Day 11 embryos. The data are plotted as the percentage change from the stabilized rate achieved after the administration of the previous drug in the sequence. The Day 11 embryos all had more than 20 somites. The Day $10^1/_2$ embryos all had less than 16 somites. Vertical error bars correspond to the standard error of the means of each set (see Table I). The *P* indicates the probability of the difference between the two groups being random as determined by Student's *t* test.

response of the reduced Day $10^1/_2$ set of embryos to carbamylcholine and the marginally significant response to atropine is consistent with the interpretation that the parasympathetic receptor of the heart is not yet functioning.

There was no difference between the response of the two groups of embryos to the isoproterenol and both sets were clearly responsive. This result is consitent with the result reported in a previous paper (6).

We interpret the insensitivity of the embryos as a reflection of the absence of innervation and nicotinic-cholinergic receptors at this stage of development. The absence of any heart rate response to low doses of nicotine would make the presence of a ganglionic nicotinic mechanism unlikely.

Hall (4) reported the results of experiments with excised embryonic rat hearts and heart fragments. He observed that at concentrations up to 100 μg/ml of medium hearts from Day $10^1/_2$ embryos were insensitive to ACh but that hearts from Day $11^1/_2$ to Day $14^1/_2$ embryos were sensitive and exhibited marked bradycardia. The decrease in heart rate was progressively larger as the age of the embryos increased. Wildenthal's (13) results with mice of gestational age 13 days and older was similar. He studied isolated hearts of mice from Day 13 to term and observed progressively greater bradycardia in response to ACh. A mouse at Day 13 of gestation is approximately at the same stage of development as a rat at Day 14.

On the other hand, Pager *et al.* (5) observed no effect with ACh on excised embryonic hearts at Day $13^1/_2$ at concentrations up to 100 μg/ml of medium. Their observations were with Days $13^1/_2$, $16^1/_2$, and 20–21 with doses larger than 1 μg/ml. Their experiments were done at 20°C in contrast to Hall's and ours which were done at 37.5°C.

Pager *et al.* explain the lack of response to ACh which they observed in the Day $13^1/_2$ hearts by differences in temperature and ionic composition of the medium. However, the ionic composition of the Krebs–Ringer

solution used by Hall and the medium used by Pager *et al.* are not very different. The lower temperatures used by Pager *et al* may be the most significant factor. Robkin *et al.* (9) have shown that the heart rate of intact rat embryos drops with decreasing temperature at a rate of about 7% per degree. Thus the heart rate at 24°C would be almost a factor of 3 smaller than the rate at 37.5°C. The lower temperature may have slowed the hearts to the point where further slowing due to ACh could not be manifested. In any event, 24°C is not a physiological temperature for either mammalian embryos or incubating chicks.

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