ONTogenESIS OF CHOLINERGIC INNERVATION IN THE RAT HEART

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SUMMARY We studied the developmental sequence of transmitter synthesis and neurotransmission by parasympathetic neurons in rat heart. Measurements of atrial and ventricular choline acetyltransferase activity were used as indicators of acetylcholine synthesis, and negative chronotropic effects of neuronal stimulation were used to demonstrate neuroeffector transmission. Activity of the mitochondrial enzyme, carnitine acetyltransferase, was measured separately to avoid spuriously high apparent choline acetyltransferase activity. Activity of the acetylcholine synthetic enzyme, choline acetyltransferase, first was detected in rat hearts on embryonic day 19, and the levels of the enzyme activity increased during the first and third weeks after birth, with levels in the atria always appearing higher than in the ventricles. In contrast, carnitine acetyltransferase activity was higher in the ventricles than in the atria, and the activity levels increased continuously from embryonic day 18 to postnatal day 21, the last day measured. Neuroeffector transmission first appeared in the rat heart on embryonic day 21, the day of birth, and was blocked by atropine. Newborn and adult hearts had similar acetylcholine sensitivity (ED₅₀ of 100 nm). Embryonic rat hearts showed slightly higher acetylcholine sensitivity. The avian class was used for comparison, and choline acetyltransferase activity first appeared in embryonic chicks on day 11. The pattern of neurotransmitter synthesis and function was identical in the avian and mammalian species, although the mammal is less mature at birth. Our studies have established the time course for development of the synthetic enzyme for acetylcholine and subsequent neurotransmission in the rat heart. Circ Res 46: 690-695, 1980

INNervation is a primary factor causing post-junctional changes in the membrane characteristics of muscle cells (Guth, 1968; Gutman, 1969; Fleming et al., 1973). During avian heart development, changes of the myocardial cell membrane in resting and action potentials (Sperelakis, 1972), ionic channels (Sperelakis and McLean, 1978), muscarinic cholinergic binding sites (Galper et al., 1977), and sensitivity to cholinergic agents (Löf felholz and Pappano, 1974) occur at the commencement of cholinergic neurotransmission. Before the trophic role of cholinergic innervation can be related to these developmental alterations, the time course of ontogenesis of cholinergic innervation needs to be established. The development of innervation is more accurately followed by studying the evolution of neurotransmitter synthesis and subsequent functional neuroeffector transmission (Pappano and Löf felholz, 1974; and Pappano, 1975) than by the anatomical appearance of neurons within the myocardium. Although the development of functional innervation in the avian heart has been examined (Pappano, 1975; and Pappano, 1977), the ontogenesis of cholinergic innervation in the mammalian heart has not been explored fully. This study was designed to establish the age-related appearance of neurotransmitter synthesis and neuroeffector transmission in cholinergic neurons in the developing rat heart. Atrial and ventricular choline acetyltransferase activity was measured during cardiac development as an indicator of the capacity to synthesize acetylcholine. Cardioinhibition in response to direct electrical stimulation of cholinergic neurons demonstrated the establishment of neuroeffector transmission. Avian cardiac muscle also was examined to compare the pattern of development of avian choline acetyltransferase activity to that of the mammal.

Methods

Animal Source

Embryonic and neonatal rat hearts were taken at the designated ages from the Wistar colony at the University of Iowa. Early morning vaginal smears were examined microscopically, and the presence of spermatozoa was considered as positive for concep-
tion and recorded as gestational day zero. Embryonic chick hearts were removed at specified days from incubated fertilized eggs of White Leghorn hens, supplied by SPAFAS Inc.

The hearts were dissected to remove the following tissue for enzymatic assay: (1) the free wall and appendage of the right atrium, including the sino-atrial node area and excluding the atrial septum and tricuspid valve; (2) the free wall and appendage of the left atrium, excluding the atrial septum and mitral valve; (3) the sinus and infundibular portions of the right ventricle, excluding the ventricular septum and tricuspid and pulmonary valve tissue; and (4) the sinus and outflow tract portions of the left ventricle, including the ventricular septum and excluding mitral and aortic valves. After these myocardial sections had been rinsed in Earle’s solution (0-4°C) to remove adherent blood, they were frozen and stored in liquid nitrogen.

Cell Cultures

Primary cell cultures of newborn to 4-day-old rat ventricles were prepared and maintained as previously reported (Marvin et al., 1979). Under aseptic conditions, the ventricles of 10-15 rats were removed and placed in a petri dish containing CV2M wash solution with penicillin, 50 U/ml, streptomycin, 50 μg/ml, and gentamicin, 100 μg/ml, added. The CV2M solution contained 50% M199, 45% Earle’s balanced salt solution, and 15% horse serum. After two rinses to remove blood, the ventricles were minced with fine dissecting scissors into fragments approximately 1 mm in diameter. The ventricular fragments then were serially exposed to eight 30-minute incubations in trypsin solution at 37°C with stirring. The trypsin solution contained trypsin, 1 mg/ml (1:250, ICN Pharmaceuticals Inc.) in isotonic solution based on Na+ and K+ (ISNK) (composition in mm/liter): NaCl, 133; KCl, 4.7; glucose, 16.5; HEPES, 20.0 (Calbiochem), and phenol red, 0.014. Approximately eight incubation periods were examined for neurotransmitter sensitivity, including mitral and aortic valves. After these myocardial sections had been rinsed in Earle’s solution (0-4°C) to remove adherent blood, they were frozen and stored in liquid nitrogen.

Enzyme Determinations

Choline acetyltransferase activity and carnitine acyltransferase activity were assayed as previously reported (Roskoski et al., 1974; Roskoski et al., 1975; and Roskoski et al., 1977). Rat or chick heart tissue was homogenized in 250 μl of potassium phosphate buffer (pH 7.4) containing 0.1 mm EDTA, using a glass-to-glass Duall tissue grinder (Kontes). [14C]Acetylcholine was resolved from acetylcholine, acetyl-CoA, and other metabolites by low voltage (35 V/cm) paper electrophoresis (Whatman no. 1) in a Gelman deluxe paper electrophoresis apparatus. Radioactivity of the specified compounds was measured as previously described (Roskoski et al., 1974). Activity of samples with less than 100 counts/min (cpm) was considered negligible, since under our experimental conditions the background was 70 cpm, including the 30-cpm machine background. Using 55 mCi [14C]acetyl-CoA per mmol (New England Nuclear Corp.), we determined that 1 pmol of radioactivity was 73.3 cpm on the Beckman LS 100. Therefore, the limit of detection in an aliquot containing 10 mg protein/ml was approximately 0.25 pmol/min per mg protein (15-minute incubation) to 0.06 pmol/min per mg protein (60-minute incubation). Enzyme activity was based on protein determined by the method of Lowry et al. (1951).

Neuroeffector Transmission

The carotid sheaths of designated embryonic and neonatal rats were exposed by a transverse cervical incision. The right and left vagi were bluntly dissected free of the carotid sheaths and directly stimulated with 0.5-msec rectangular pulses at 5 V and 10 Hz for 5-10 seconds through fine Pt wires. The epicardial surface of the rat heart was exposed just prior to stimulation via a median sternotomy, and heart rate was determined by inspection or recorded by strain gauge on a Grass polygraph. The appearance of a cardioinhibitory response (decrease in heart rate) to stimulation was considered evidence of neuroeffector transmission only if the heart rate returned to baseline after stimulation and if the addition of atropine (10-6 m, Sigma) blocked the cardioinhibitory response.

Acetylcholine Sensitivity

Designated embryonic and neonatal rat hearts were examined for neurotransmitter sensitivity about the time that neuroeffector transmission was found to occur. Hearts were removed and placed in a muscle chamber and superfused with ISKO (composition in mm/liter: NaCl, 122.7; NaHCO3, 16.0; CaCl2, 1.8; NaH2PO4, 1.0; dextrose, 5.5; and HEPES buffer, 20.0, at pH 7.3) combined with M199 in a 1:1 mixture, yielding a K+ concentration of 2 mM. Acetylcholine chloride (Sigma) in varying concentrations was applied as a pulse injection by Eppendorf micropipette into the muscle chamber (see Hermens and Robinson, 1977, for further details). The resulting cardioinhibitory response was monitored stereomicroscopically or recorded with microelectrodes. Heart rate or action potential frequency was

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observed during the first 15 seconds after pulse application (the time of maximum inhibition). The mean data from three applications of a given acetylcholine concentration constituted the accepted chronotropic response. Return to baseline rates with control ISKO and M199 superfusion and blockade of inhibition with pulse applications of atropine ($10^{-6}$ M) were our criteria to exclude artifactual cardioinhibitory responses.

All data were analyzed by Student's group t-test comparisons. $P$ values less than 0.05 were accepted as significant.

**Results**

**Choline and Carnitine Acetyltransferase Activity in Prenatal and Neonatal Rat Hearts**

We first detected significant choline acetyltransferase activity, ranging from 2 to 8 pmol/min per mg protein, in both the atria and ventricles on the 19th gestational day (Fig. 1). Although atrial activity was consistently higher than ventricular activity, a significant difference was most readily apparent after birth. Ventricular activity reached 12 pmol/min per mg protein during the first week of neonatal life. Unlike the ventricular enzyme activity, atrial enzyme activity increased to 30 pmol/min per mg protein by the end of the second week before reaching a steady state value.

In contrast to these results, no choline acetyltransferase activity was found in the primary cell cultures of neonatal rat ventricles ($n = 20$). To exclude the possibility of myocardial deterioration during culture preparation as an explanation for undetectable choline acetyltransferase activity, carnitine acetyltransferase activity was measured. The latter enzyme activity was about 1000-fold higher than the former enzyme activity in the developing rat heart (Fig. 2). The level of carnitine acetyltransferase activity was $7.5 \pm 1$ (n = 20) nmol/min per mg protein in the cultured ventricular muscle cells, a value comparable to that in source hearts. The carnitine acetyltransferase activity in ventricles always was higher than in the atria. Choline acetyltransferase activity was present in the supernatant after centrifugation during the culture preparation; the supernatant contained the neuron fragments and vesiaces. The muscle cells in the pellet, on the other hand, contained no choline acetyltransferase activity.

**Choline and Carnitine Acetyltransferase Activity in Prenatal Chick Hearts**

The time course of choline acetyltransferase ontogenesis was determined also in embryonic chick hearts. The larger mass of the embryonic chick heart, compared to the embryonic rat heart of the same gestational age, permitted determinations of single rather than paired right and left cardiac chambers. Choline acetyltransferase activity was not measurable prior to the 11th gestational day, after which a level of approximately 5-10 pmol/min per mg protein appeared in all four cardiac chambers (Fig. 3). After 3 days, a significantly different plateau level was apparent between the atria, 28-35 pmol/min per mg protein, and the ventricles, 8-11 pmol/min per mg protein. No significant differences in enzyme activity were apparent between the right and left atrial or ventricular chambers. Carnitine acetyltransferase activity was approximately 1000-fold higher than choline acetyltransferase activity (Fig. 4). Carnitine acetyltransferase activity rose rapidly from embryonic day 10-15 in all four chambers and continued to increase to hatching age.
The Development of Neuroeffector Transmission in the Rat

The onset of neuroeffector transmission was determined by direct electrical stimulation of the right and left cervical vagal nerves in vivo. Experiments were initiated in rats during the 18th gestational day, the oldest age at which choline acetyltransferase activity was undetectable. Vagal stimulation, even up to 100 V, failed to elicit a cardioinhibitory response before the 21st gestational day in 36 rats, even though significant levels of choline acetyltransferase activity were found on the 19th and 20th gestational days (Fig. 3). The mean spontaneous contraction frequency was 165/min. On the 21st gestational day, vagal stimulation consistently resulted in a 30–60% reduction in spontaneous contraction frequency (n = 24). This reduction in frequency could be repeated for only two to three periods of 10 seconds in each animal. The short duration (0.5 msec) pulse and complete inhibition by 1 mM atropine ensured that nerve acetylcholine release, rather than muscle field stimulation, produced the decrease in spontaneous heart rate.

The sensitivity of the intact heart to exogenous acetylcholine from embryonic day 12 to the onset of neuroeffector transmission was investigated. Dose-response curves showed that the ED50 is about 17 nM at day 12, but significantly higher thereafter (Fig. 5). Data points for 15-, 18-, and 21-day embryonic hearts were not significantly different, allowing pooling of data to give the other curve (ED50 = 100 nM). At acetylcholine concentrations below 100 nM, atropine (1 mM) completely abolished the bradycardia.

Discussion

We have described the development of cholinergic innervation of the heart by determination of the enzyme essential for the synthesis of acetylcholine and by demonstrating the initial appearance of vagal function. We believe that choline acetyltransferase activity serves as a useful indicator of vagal innervation. Two lines of investigation support the notion that myocardial cells per se lack the enzyme choline acetyltransferase. First, we previously found that innervated chick heart contained enzyme activity which could be separated from the heart muscle cells after trypsinization and centrifugation, and that the muscle cells (in culture) lacked the enzyme activity (Roskoski et al., 1977). Second, cardiac denervation associated with transplantation (in rats) produced a selective loss of choline acetyltransferase (from 50 to 98%, depending on the region), but not of the mitochondrial enzyme, carni-
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Atrial choline acetyltransferase (Lund et al., 1978). These conclusions differ from an earlier report which proposed myocardial cells as a source of choline acetyltransferase (Gifford et al., 1973). We believe their results were confused by contamination with carnitine acetyltransferase activity, which they did not separate from choline acetyltransferase.

Organogenesis of the rat heart proceeds from the 9th to the 15th embryonic day (New, 1973). Anatomic (Gomez, 1958) and histochemical studies with acetylcholinesterase (Navaratnam, 1965) have demonstrated parasympathetic neurons within the rat myocardium during cardiac organogenesis. We found a delay between the reported appearance of cholinergic neurons within the myocardium and the detection of the neuronal enzyme essential for the synthesis of neurotransmitter (day 19). Such a delay is necessary when the target organ contact with neurons modulates differentiation of a neurotransmitter mechanism (Bunge et al., 1978). This influence of muscle on nerve might be called the "musculotropic influence." Furthermore, a second delay appeared between the detection of choline acetyltransferase activity and the onset of neurotransmission on the 21st day. Other authors have suggested that this second delay is a result of preregional buildup of neurotransmitter (Pappano and Löffelholz, 1974; Pappano, 1975).

The identification of the neuronal source of choline acetyltransferase activity, as pre- or postganglionic, was not possible with our assay. However, if only preganglionic choline acetyltransferase activity was detected, neuroeffectation transmission would be absent, since the postganglionic neuron must be operative for vagal inhibition. On the other hand, the preganglionic neuron could not have developed much earlier than the postganglionic neuron or else choline acetyltransferase activity would have been detected earlier. Thus, our data indicated the development of pre- and postganglionic choline acetyltransferase activity was at about the same time.

Qualitative and quantitative comparisons of cholinergic ontogenesis in the embryonic rat and chick hearts revealed striking similarities. The chronologic sequence of cholinergic innervation in the embryonic chick heart has been reported as: (1) the appearance of cholinergic neurons within the myocardium on day 4 (Romanoff, 1960); (2) the earliest detectable release of acetylcholine on day 10 (Pappano and Löffelholz, 1974; Pappano, 1975); and (3) vagal neurotransmission on day 12 (Pappano, 1975). We detected choline acetyltransferase activity in embryonic chick hearts on day 11, strengthening the support for the time course proposed by Pappano and indicating that choline acetyltransferase activity is an appropriate marker for cholinergic innervation. In the rat and chick, choline acetyltransferase activity was detected simultaneously in atria and ventricles. Atrial choline acetyltransferase activity increased more than 2-fold above ventric-

ular enzyme activity, a manifestation of denser atrial parasympathetic innervation. Therefore, avian and mammalian cardiac cholinergic innervation followed an identical course related to their pattern of development. The disparity between gestational age of embryos at the time of appearance of neurons, choline acetyltransferase activity, and neurotransmission reflected the lower maturity of the mammalian heart at each age.

Sensitivity of embryonic hearts to acetylcholine was high in our experiments. We have reported the high sensitivity to norepinephrine of rat myocardial cells in primary culture (Marvin et al., 1979) without adrenergic neurons (Marvin et al., 1977). Our finding of higher acetylcholine sensitivity of rat hearts prior to innervation is similar to our findings in cultured rat myocardial cells (unpublished observations). Desensitization of cholinergic receptors appears to be important in hearts prior to innervation, making sensitivity measurement very dependent on experimental conditions (Hermesmeyer and Robinson, 1977). We believe that an earlier report of increasing sensitivity to acetylcholine with embryonic age (Hall, 1957) may be explained by desensitization phenomena with constant exposure to acetylcholine.

In summary, the present data outline the ontogenesis of cholinergic innervation in a mammalian heart. In the rat, as in the chick, neurotransmitter function begins long after the first anatomical evidence of parasympathetic innervation. This delay, dependent on the synthesis of neurotransmitter, may be a common feature in the cholinergic innervation of vertebrate hearts. Therefore, we conclude that the onset of neuroeffectation transmission, rather than the anatomical appearance of intracardiac neurons, marks the beginning of neurotrophic modulation of cardiac development.

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