Vagus Nerve Stimulation Alters Regional Acetylcholine Turnover in Rat Heart

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SUMMARY. The turnover of neurotransmitter is a direct measure of neuronal function, varying with the impulse activity of the nerve. It is not known if vagal stimulation increases acetylcholine release uniformly throughout the heart, or if modification of neural signals occurs between the vagal nerve trunks and postganglionic synaptic terminals. The rate constant of acetylcholine turnover was measured in conduction and contractile regions of heart by quantifying the incorporation of [3H]choline into acetylcholine after labeling of the blood choline pool in urethane-anesthetized rats during two levels of vagal activity. Choline and acetylcholine were assayed by high pressure liquid chromatography with electrochemical detection of post-column enzymic reaction product, peroxide. The specific activities of choline and acetylcholine in the tissues at sacrifice were used to calculate the fractional turnover rates in cardiac regions. Supramaximal bilateral vagal stimulation for 20 minutes decreased heart rate (P < 0.05), while mean arterial blood pressure remained constant. The rate constants for acetylcholine turnover in right atrial regions containing the sinus atrial node, left atrial tissues, and interatrial septum doubled from control values during vagal stimulation. In contrast, the fractional rate constants of acetylcholine turnover did not change in the right and left ventricles during vagal stimulation. We interpret these results to indicate general activation of postganglionic parasympathetic fibers to the atria and selective modulation of postganglionic parasympathetic neural function to the ventricles. (Circ Res 58: 372-377, 1986)

Vagal innervation of the heart has been studied indirectly by assay of the metabolic enzymes of synthesis, choline acetyltransferase (CAT) (Roskoski et al., 1975; Ekstrom, 1978), and degradation, acetyl cholinesterase (Tucek and Vlk, 1962; Jacobowitz et al., 1967). These measurements allow the characterization of normal distribution of cardiac vagal innervation (Roskoski et al., 1974; Sinha et al., 1976; Schmid et al., 1978) and alterations following vagotomy (Brown, 1976; Lund et al., 1979; Brown, 1981), transplantation (Lund et al., 1978), and hypertrophy and heart failure (Roskoski et al., 1975). These measurements are indices of nerve density (Roskoski et al., 1977), however, and do not reflect the functional status of the parasympathetic system.

Measurements of acetylcholine and choline allow an alternative assessment of innervation (Brown, 1976; Brown et al., 1985). Acetylcholine has a non-uniform regional pattern of concentration in the heart (Oda et al., 1984; Brown et al., 1985). In the hearts of cats after unilateral vagotomy, arithmetic addition of the decrease in tissue acetylcholine concentrations (Brown, 1981) did not result in decreases in acetylcholine concentration found after bilateral vagotomy (Brown, 1976). Furthermore, in transplanted rat heart, acetylcholine concentrations, while decreasing with regional non-uniformity, did not decrease to the extent of previously reported decreases in CAT activity (Lund et al., 1979; Oda et al., 1984). Thus, measurement of acetylcholine content or choline acetyltransferase activity also appears to be of limited value for assessing the functional status of cardiac parasympathetic innervation.

In this study, we investigated the possibility that acetylcholine turnover, determined by measuring the rate of incorporation of labeled choline into labeled acetylcholine, provides an index of regional cardiac parasympathetic nerve function (Macintosh and Collier, 1976). Brown and Salata (1983) recently reported the determination of acetylcholine turnover rates in resting whole rat heart. In the present study, we report differences in fractional rate constants for acetylcholine turnover in discrete regions of rat heart during both basal and vagal-stimulated states.

Regional variation in fractional rate constants of acetylcholine turnover under conditions of controlled vagus nerve stimulation might reflect variable modulation of neurotransmission at either ganglia or neuroeffector junctions. Our data suggests there is selective modulation of parasympathetic innervation to cardiac ventricles, in comparison to cardiac atria.

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Methods

Male Sprague-Dawley rats (225–260 g) were anesthetized with urethane (1.2 mg/kg, ip). Cannulas were placed in the femoral artery to allow measurement of blood pressure and blood sampling, and in the femoral vein to allow injection of $[^{3}H]$choline (0.1 mCi/kg, 20 μM, bolus, iv). Both cervical vagi were isolated, but left intact; bipolar stainless steel electrodes were placed on both vagi. Stimulation parameters were: current, 0.5 mA; frequency, 15 Hz; duration, 2 msec; period of stimulation, 20 minutes. Before each experiment, a stimulus-response relationship with heart rate response was determined to establish supramaximal stimulation of the vagi; rats were allowed to recover the basal hemodynamic states (about 20 minutes) before labeled choline was injected and the 20-minute experimental period was begun. Vagi from control animals were isolated and placed on the electrodes, but were not stimulated during the assessment of acetylcholine turnover. Blood pressure was recorded throughout the experiment; heart rate was obtained from the phasic pulsation of the blood pressure tracing.

Blood samples (0.8 ml) were withdrawn 5, 10, 15, and 20 minutes after the injection of $[^{3}H]$choline (length of time of injection, 0.22 ± 0.01 minute, n = 11) to follow the decrease in plasma choline specific activity. Fluid was replaced with physiological saline to prevent alterations in blood volume. Plasma (0.4 ml) was separated from red cells by centrifugation; plasma samples were mixed with 0.1 volume 4% perchloric acid and chilled for 15 minutes, before the precipitated proteins were separated by centrifugation in an Eppendorf microfuge at 12,500 g for 5 minutes. The acid supernatant was neutralized by addition of 0.1 volume 7.5 M potassium acetate, which also removed perchlorate ion as the potassium salt. The neutralized, deproteinized supernatant was separated by centrifugation and frozen for later analysis of choline.

Rats were killed at 20 minutes, and heart samples (right and left atrial appendages, interatrial septum, and left and right ventricular free walls) were obtained for acetylcholine analysis. The stability of the neurotransmitter pool postmortem was tested by measuring the remaining choline acetyltransferase and acetylcholinesterase activities in samples subjected to varying periods of time of microwave irradiation in 0.5 ml saline. The optimal time chosen was 10 seconds, as both enzyme activities had decreased to less than 1% of initial activity, without an observed decrease in acetylcholine levels. The samples were microwave-irradiated, blotted, weighed, and frozen in liquid nitrogen; the time from sacrifice to freezing was less than 2 minutes. Samples were stored in a −20°C freezer until homogenization for acetylcholine analysis. Frozen samples have shown no changes in acetylcholine levels over several months.

Tissues were homogenized in 5% trichloroacetic acid in neat acetonitrile, and the supernatant was dried under a stream of air at 40°C. The samples were resuspended in 1 mM HCl (0.5 ml), and choline and acetylcholine were isolated by silica column (0.8 mm i.d. × 0.7 mm height; 350 μl) elution (Gilberstadt and Russell, 1984), both being eluted together with 1.5 ml 30% HC1 containing 10% 2-butanone (methyl ethyl ketone). The eluate was dried in a vacuum centrifuge, and stored in a −20°C freezer.

Samples were dissolved in 10 mM sodium acetate, pH 3.5; injection volume onto the HPLC was 20 μl. After separation by reverse-phase column chromatography, choline and acetylcholine were analyzed by electrochemical detection of peroxide generated by post-column enzymatic reaction with acetylcholinesterase and choline oxidase (Potter et al., 1983). The chromatographic column was 2.0 × 150 mm, 5 μm ODS (Alltech Assoc.), protected by a similar guard column (5 μm ODS, Bio-Rad). The separation buffer was 10 mM sodium acetate/citric acid, pH 5.0, containing 1 mM tetraethylammonium chloride and 30 mg/liter sodium octyl sulfate with 250 μl phenol as a bacteriostat. Flow rate was 0.3 ml/min. The column was maintained at 55°C; back-pressure was under 3000 psi. Enzyme buffer, 0.1 M glycylglycine, pH 8.2, containing 800 μl/liter choline oxidase (no. 5896, Sigma), 200 μl/liter acetylcholinesterase (no. 2888, Sigma) and 100 μl phenol, was pumped by a Milton Roy model 396 pump at the rate of 0.2 ml/min. A 10-m coil of 0.3 × 0.6 mm (30 gauge) Teflon connected the mixing "T" and the detector cell; back-pressure through the detection system (LC-4B amperometric detector with TL-10A Pt electrode, and RE-3 reference electrode; BioAnalytical Systems) was about 20 psi. Detector voltage was set at 0.500 V vs. a Ag/AgCl reference electrode. Under these conditions, choline eluted at 4.9 minutes, and acetylcholine at 10.4 minutes relative to the injection time. Sensitivity was about 3 pmol of each compound injected onto the column; normally, standards in the range of 10–1000 pmol were used. The recovery of choline standards, carried from homogenization to analysis, was 98 ± 1.7% (man with SEM, n = 8); that of acetylcholine was 96 ± 2.2% (n = 8). Samples were appropriately corrected for these recoveries. The detector eluate was fractionated and collected. The radioactivity under the choline and acetylcholine peaks was quantified by liquid spectrometry.

The rate constant of choline utilization was computed from the decline of plasma choline specific activity over time. Using this derived value and the specific activities of tissue choline and acetylcholine, we calculated fractional rate constants of acetylcholine turnover, $k_{ACh}$, from the equation:

$$[ACh] = \frac{K}{k_{Ch}} \left[1 + \frac{(k_{ACh}e^{-k_{ACh}t} - k_{ACh}e^{-k_{Ch}t})}{(k_{ACh} - k_{Ch})}\right]$$

where $[ACh]$ is tissue acetylcholine specific activity, $K$ is the maxima of the plasma choline specific activity curve, $k_{Ch}$ is the rate of plasma choline disappearance, $k_{ACh}$ is the rate of acetylcholine synthesis, and $t$ is the time of sacrifice (from injection of the labeled choline). A modification of a norepinephrine turnover computer program (Schmid et al., 1984) was used to solve for the integrated rate constant of acetylcholine synthesis over the entire 20-minute period from the decline of the plasma choline specific activity, and the specific activities of tissue choline and acetylcholine at the time of sacrifice. A copy of these programs are available from our laboratory on request.

Two statistical analyses were done. "T-statistics" for each tissue region were made between control and stimulated samples based on the "within-cell mean square" computed from the analysis of variance (Winer, 1962). Second, a "within-group" multiple comparison was made with the Bonferroni multiple comparison procedure (Neter and Wasserman, 1974).
Results

Blood pressure and heart rate were continually monitored throughout the experimental protocol. Blood pressure remained unchanged throughout the experiment (Fig. 1). Supramaximal, bilateral vagal stimulation dramatically decreased heart rate (Fig. 2), which initially decreased to 24% of control. The heart rate increased slowly throughout the duration of the experiment, increasing to 38% of control at 10 minutes, and 45% at the end of the 20-minute experiment.

After bolus injection of radiolabeled choline, the specific activity of the plasma choline pool decreased exponentially with time (Fig. 3). The calculated rate of disappearance of labeled choline from the plasma pool was 2.10 ± 0.18/hour for control animals; this rate doubled to 4.54 ± 0.50/hour during vagal stimulation (P < 0.05). The plasma concentration of choline was not altered by the stimulation; it varied less than 10% in both control and stimulated animals during the period of experimentation (control, n = 6, 21.4 ± 1.4 vs. stimulated, t = 20 minutes, n = 5, 23.2 ± 2.1 nmol/ml plasma).

Under basal conditions, there was regional variation of acetylcholine concentration in the four chambers of the rat heart (Table 1). Acetylcholine concentration was highest in right atrial tissue containing the sinoatrial node region, intermediate in left atrium and interatrial septum, and lowest in ventricles. There were no changes in acetylcholine concentration between basal and stimulated groups when compared by region.

<table>
<thead>
<tr>
<th>Region</th>
<th>Control</th>
<th>Stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right atrium + SA node</td>
<td>19.5 ± 2.0</td>
<td>17.1 ± 1.8</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(n = 5)</td>
<td></td>
</tr>
<tr>
<td>Left atrium</td>
<td>7.64 ± 0.77*</td>
<td>8.53 ± 1.26*</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(n = 5)</td>
<td></td>
</tr>
<tr>
<td>Interastral septum</td>
<td>9.24 ± 0.87*</td>
<td>9.12 ± 0.70*</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(n = 4)</td>
<td></td>
</tr>
<tr>
<td>Right ventricle</td>
<td>1.21 ± 0.20†</td>
<td>1.36 ± 0.35†</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(n = 4)</td>
<td></td>
</tr>
<tr>
<td>Left ventricle</td>
<td>1.51 ± 0.08†</td>
<td>1.69 ± 0.31†</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(n = 4)</td>
<td></td>
</tr>
</tbody>
</table>

There were no significant differences within a cardiac region between control and stimulated rats.

* P < 0.05, compared to right atrium.
† P < 0.05, compared to right atrium, left atrium, interatrial septum.
Fractional rate constants for acetylcholine turnover also varied regionally in hearts of rats under basal conditions (Fig. 4). The values for these rate constants varied from about 3.5/hour in the atria to less than 2.0/hour in the ventricles. In response to vagal stimulation (Fig. 4), rate constants of acetylcholine turnover increased in right atrium, left atrium, and interatrial septum. The fractional rate constant was unchanged in right ventricle and left ventricle. Acetylcholine synthesis, calculated as the product of acetylcholine concentration and fractional rate constant of acetylcholine turnover, varied regionally in the rat heart (Fig. 5). Acetylcholine synthesis was highest in the right atrium (63 ± 12 nmol/g per hour), intermediate in the left atrium and interatrial septum (27 ± 3 and 27 ± 5 nmol/g per hour, respectively), and lowest in ventricular tissues (right ventricle, 2.3 ± 0.3 nmol/g per hour; left ventricle, 2.6 ± 0.4 nmol/g per hour). The rank order was: right atrium > left atrium = interatrial septum > right ventricle = left ventricle. Vagal stimulation doubled acetylcholine synthesis rates in the atrial tissues (P < 0.05), and did not affect synthesis rates in the ventricles (Fig. 5).

Discussion

This is the first report of regional differences in cardiac acetylcholine turnover and synthesis. Furthermore, vagal stimulation was shown to cause disparate regional changes in the fractional rate constant of acetylcholine turnover in rat heart. Supramaximal bilateral vagal stimulation doubled the rate constant of acetylcholine turnover in the right atrium, left atrium, interatrial septum, whereas there was no change in the fractional rate constants in the right and left ventricles. Acetylcholine synthesis rates, calculated as the product of regional acetylcholine concentration and fractional turnover rate constant, increased in atrial tissues with vagal stimulation, but remained unchanged in ventricular tissues.

Acetylcholine concentrations varied in discrete regions of rat hearts. Nonuniform regional neuronal indices have been a consistent feature of cardiac autonomic innervation, being observed in the sympathetic system (Schmid et al., 1979) and the parasympathetic system. Acetylcholine concentration was highest in the right atrium containing the sinoatrial node, intermediate in the left atrium and interatrial septum, and lowest in the contractile tissues of the ventricles (Brown et al., 1985). This pattern is consistent with the distribution of CAT activity (Lund et al., 1978, 1979), which as been interpreted to reflect neuronal density (Roskoski et al., 1977).

A large difference exists between the activity of the biosynthetic enzyme, choline acetyltransferase, determined in vitro (Lund et al., 1978, 1979), and the synthesis rates determined in vivo in this study. The reasons for these differences are not readily apparent; the large excess of enzyme activity over acetylcholine synthesis (10-fold in atria and 100-fold in ventricles) might reflect optimal in vitro assay conditions (pH, temperature, and saturating concentrations of both substrates, Ch and acetyl coenzyme A), which might not exist in vivo. Comparable differences between enzyme activity and acetylcholine synthesis are noted in brainstem areas (Jenden, 1979).

The fractional rate constant is a dimensionless term which refers to that fraction of the endogenous pool of neurotransmitter that is utilized and replaced by de novo synthesis during a given time period (Zilversmit, 1960). The fractional rate constant is independent of concentration or density of innervation, but is tightly coupled to impulse conduction and neurotransmitter release (MacIntosh and Collier, 1976; Moroni et al., 1978). As such, it is an
Basal fractional rate constants of acetylcholine turnover vary throughout cardiac regions. The fractional rate constants under basal conditions show the same distribution pattern as the activity of choline acetyltransferase. The biosynthetic enzyme, determined in vitro (Lund et al., 1978, 1979; Brown et al., 1985) and 376 no ganglionic cells. The increased acetylcholine turnover in atria during vagal stimulation is consistent with the observed decrease in heart rate. The absence of detectable change in acetylcholine turnover in right and left ventricles is consistent with modification of neurotransmission between the point of vagal activation and postganglionic synapses in cardiac ventricles.

Regional variation in acetylcholine turnover to vagal stimulation may result from modulation of neurotransmission at either the ganglionic synapse or the postganglionic neuroeffector junction. Control of vagal activation by supramaximal vagal stimulation allowed detection of peripheral modulation of responses in cardiac ventricles. The observed lack of change in ventricular fractional rate constants with vagal stimulation might be due to selective inhibition of cholinergic activity at the ganglionic level. Reciprocal modulation between sympathetic and parasympathetic cardiac innervation (Levy, 1971, 1984), or attenuation of postganglionic parasympathetic signals by regulatory neurons, might account for selective inhibition of ventricular acetylcholine turnover during vagal stimulation. α-Adrenergic-mediated inhibition of acetylcholine release from heart has been suggested (Starke, 1972), and recently demonstrated as a mechanism in rat atria (Wetzel and Brown, 1985; Wetzel et al., 1985).

Intraganglionic cholinergic mechanisms may also play a role in modulation of the preganglionic signal. Activation of ganglion cells with axon collaterals, which have been demonstrated both functionally (Roper, 1976) and visually (McMahan and Purves, 1976) in amphibian cardiac ganglia, may be responsible for modulation of ganglion cells. Ganglionic muscarinic receptor sites, shown to produce inhibitory postsynaptic potentials in cardiac ganglion cells of amphibians (Hartzell et al., 1977), may be activated by excessive vagal stimulation and inhibit postganglionic nerve activity.

At the postganglionic neuroeffector junction in cardiac ventricles, regulatory mechanisms which could inhibit acetylcholine turnover during vagal activation might include alterations in release of acetylcholine by co-transmitters (e.g., γ-aminobutyric acid of substance P) or endogenous substances, such as adenosine metabolites (Stone, 1981) and differences in muscarinic receptor subtype (Barlow et al., 1976; Loeb et al., 1982). Alternatively, reflex sympathetic activity might lead to an increased local concentration of norepinephrine in the ventricular perfusate (Loeb et al., 1984). Local modification in relative proportions of cholinergic and nonadrenergic neurotransmitters released by ventricular postganglionic neurons could have profound influence on the interaction of the two systems at the neuronal terminal.

This study has confirmed regional differences in acetylcholine concentrations and revealed regional differences in acetylcholine turnover in rat heart. Most strikingly, we have shown disparate regional changes in fractional turnover rates of acetylcholine. Our data showing increases in the fractional turnover rate constant of acetylcholine synthesis in atrial tissues and no change in the right and left ventricles with supramaximal bilateral vagal stimulation are consistent with modulation of excitatory impulse activity by peripheral mechanisms in the heart. We have also demonstrated the feasibility of assessing regional turnover rates under two different levels of vagal nerve activity. The sensitivity of the determination of fractional rate constants should be useful to investigate changes in regional acetylcholine turnover to known cardiovascular reflexes. The methods used in these studies can be extended for use in unanesthetized, free-ranging animals.

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