The chronotropic responses of isolated sinoatrial node and ventricular muscle cells to neurotransmitters were compared in vitro with and without selective adrenergic and cholinergic innervation. Explants of either thoracolumbar sympathetic ganglion or sacrococcygeal spinal cord were added to cultures of newborn rat sinus node regions or ventricular apexes harvested before the onset of autonomic innervation in vivo. Catecholamine synthesis was detected by glyoxylic acid histofluorescence. Acetylcholine synthesis was indicated by monoclonal antibody labeling of choline acetyltransferase. After electrical or pharmacological stimulation of neurons, the chronotropic response of individual myocardial cells confirmed the presence of neuroeffector transmission; the nature of the myocyte response identified the stimulated neuron as either adrenergic or cholinergic. Chronotropic responses of all myocardial cells to norepinephrine or acetylcholine were transcribed on a recorder coupled to a video photoconductive cell monitor. Isolated sinoatrial node cells were supersensitive to norepinephrine and acetylcholine; thresholds were $3 \times 10^{-16}$ M and $6 \times 10^{-15}$ M, respectively. These sinoatrial node cells remained sensitive to both norepinephrine and acetylcholine after the development of innervation in vitro. Ventricular cells also were sensitive with thresholds of $3 \times 10^{-11}$ M and $6 \times 10^{-14}$ M to norepinephrine and acetylcholine, respectively. However, following in vitro innervation, ventricular cells were significantly less sensitive to norepinephrine and acetylcholine (thresholds $3 \times 10^{-9}$ M and $6 \times 10^{-11}$ M). These data are the first to demonstrate that neurotrophic modulation is not homogeneous throughout the myocardium and that it may be dependent on the specific myocardial cell innervated. (Circulation Research 1989;64:1051-1062)
isolated ventricular muscle and sinoatrial node cells, which are morphologically and functionally comparable to their in vivo source, can be prepared. Selective adrenergic and cholinergic innervation is also feasible. This study was designed to compare the chronotropic responsiveness of single newborn rat myocardial cells, both sinoatrial node and ventricular, with neurotransmitters with or without selective adrenergic or cholinergic innervation in vitro. Isolated sinoatrial node cells were highly responsive to neurotransmitters, and this responsiveness remained predominantly unaltered after either adrenergic or cholinergic innervation. In contrast, chronotropic responses of isolated ventricular muscle cells, to both norepinephrine and acetylcholine, were diminished compared with sinoatrial cells and were further decreased after adrenergic and cholinergic innervation, respectively. Thus, neurotrophic modulation of developing myocardium may not be uniform and might be dependent upon the specific myocardial cell innervated.

Materials and Methods

Cell Cultures

Newborn rats from the Wistar-Kyoto colony at the University of Iowa were used for all cultures. As previously described,13,14 sino node regions and ventricular apexes were separately excised under aseptic conditions. The tissue fragments were placed in media consisting of 85% minimum essential medium Eagle–Earle’s salts, 15% horse serum, 4 mM L-glutamine, 10 mg/ml gentamicin, and 20 mM HEPES buffer (pH 7.3). When dissection was complete, the tissue was minced into 1–2 mm fragments and rinsed for 10 minutes in a solution containing (mM) potassium glutamate 140, HEPES 25 (pH 7.3), NaHCO3 16, NaH2PO4 0.5, dextrose 16.5, and phenol red 0.014. The tissue was dispersed by serial trypsin incubations into a cell suspension. Neurons, obtained from either thoracolumbar sympathetic chain or sacrococcygeal spinal cord, were prepared separately and simultaneously with the heart cells.15

Two or three drops of media containing neuronal fragments were placed on a single coverslip that was then incubated at 37°C with 5% CO2 for 2–3 hours, allowing attachment of the neuronal explants to the coverslip surface. When the trypsin dispersion of the cardiac cells was complete, the cell suspension was plated onto 9 × 22 coverslips in a 60 mm Petri dish at a density of 1 × 105 cells/ml. The coverslips were either empty or contained the previously seeded neural explants. All cultures were incubated at 37°C in 5% CO2. Experiments were conducted after 72 hours incubation.

Since detailed descriptions of cultures containing noninnervated and innervated ventricular muscle cells are given elsewhere, this study examined the morphology of cultures containing noninnervated and innervated sinoatrial node cells only. These cells were intermittently observed in vitro over 5 days with a Leitz Diavert microscope equipped with phase contrast or differential interference optics to assess morphology and growth patterns. Following in vitro experimentation, cultures were fixed, and neurons and neuromuscular junctions were examined with Bodian’s silver stain.

Confirmation of Neurotransmitter Synthesis

Adrenergic neurotransmitter synthesis was detected in sinus node cultures with explants by catecholamine histofluorescence using a modified glyoxylic acid technique as described previously.15,17 The glass coverslips containing the neuromuscular cultures were quickly rinsed in 0.15 M phosphate buffer (pH 7.6) at 37°C and transferred to a 2% solution of glyoxylic acid (Sigma Chemical Co, St. Louis, Missouri) in phosphate buffer (pH 7.0) for 3 minutes at 25°C. The coverslips were mounted on standard glass slides, dried for 5 minutes in room air, then heated at 100°C for 4 minutes. After sealing with a second coverslip and paraffin oil, the cells were examined for histofluorescence under a Leitz Orthoplan microscope using 490 nm incident light excitation and a 515 nm long pass filter.

Synthesis of acetylcholine in sinus node cultures with explants was indicated by monoclonal antibody labeling of choline acetyltransferase.18,19 Glass coverslips containing the neuromuscular cultures were fixed for 30 minutes in 2.0% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer followed by a 5 minute rinse in phosphate buffered saline (PBS). The cells were incubated in 0.05% Triton X-100 plus 3% normal rabbit serum (NRS) in PBS at 4°C for 1 hour. The cells were exposed to an excess of rat monoclonal anticholine acetyltransferase antibody diluted 1:500 in 3% NRS, 0.1% Triton X-100 in PBS, for 12–16 hours. After two rinses with PBS of 10 minutes each, the cells were incubated with rabbit anti–rat IgG (1:500) for 1 hour. Two 10-minute PBS rinses followed, then 1:100 peroxidase–antiperoxidase (PAP) in 3% NRS was applied to the cells for 1 hour. After two more 10-minute rinses in PBS, the exposure to the secondary antibody and PAP was repeated. Following the final PBS rinse, the coverslips were rinsed in 0.5 M Tris buffer (pH 7.6) The cells were incubated in 0.05% dianimobenzidine (DAB) in 0.05 M Tris-HCl buffer for 10 minutes at room temperature. The cells were stained at room temperature for 6–8 minutes in 0.05% DAB and 0.01% H2O2. The coverslips received a final 5-minute rinse in Tris buffer, were air dried, dehydrated with ethanol and xylene, and mounted on standard microscopic slides. Control slides were made with whole mount sections of rat brain.

Confirmation of Neuroeffector Transmission

Neuroeffector transmission in sinus node cultures with explants was confirmed by direct microelectrode stimulation in the neuromuscular cultures. A platinum electrode (0.010 in.) was placed directly...
on an explant, and a short unipolar pulse (1.5 mV, 0.5–20 Hz, 2-msec duration, 0.5-msec delay) was delivered with a Grass 544 stimulator (Grass Instruments, Quincy, Massachusetts). Notation was made of the directional change of the chronotropic response, positive or negative, of a contracting sinoatrial node cell with axons from the stimulated explant terminating on the cell surface. A neuron was considered adrenergic or cholinergic depending on appropriate acceleration or deceleration of the contracting frequencies of the sinoatrial node cell, and if continuous suffusion with 3×10⁻⁸ M propranolol or atropine, respectively, prevented the chronotropic change.

Pharmacologic stimulation also characterized the selective adrenergic or cholinergic nature of the neurons in the sinus node cultures. After recording baseline contraction frequency, either 10 ng/ml guanethidine sulfate (CIBA) or bethanechol chloride (Merck Sharp & Dohme, West Point, Pennsylvania) was applied by a 10-μl pulse. These particular neurotransmitter releasing drugs were chosen for their lack of significant primary cardiac effect. Contraction frequency was recorded during three to five successive 15-second intervals. As with electrical stimulation, the neurons were considered adrenergic or cholinergic if the contraction frequency of an individual cell increased or decreased, respectively, and if continuous suffusion with propranolol or atropine blocked this chronotropic response, respectively. Pharmacologic confirmation was preferred to electrical stimulation because it was less time consuming.

Dose-Response Curves

Glass coverslips, containing the cultured cells, were placed in an open thermally regulated suffusion chamber (200 μl) mounted on the microscope stage. The cells were continuously suffused with an O₂-CO₂ saturated solution containing (mM) NaCl 130, NaHCO₃ 16, NaH₂PO₄ 0.5, KCl 4.7, CaCl₂ 1.8, MgCl₂ 0.41, MgSO₄ 0.41, dextrose 5.5, and HEPES 13.0 (pH 7.6) through a heat-exchange bottle with a flow rate of 2 ml/min. The coverslip was quickly scanned to locate a sinoatrial node or ventricular cell with appropriate morphology and contraction frequency. The contraction frequency of either cardiac cell was visually observed for 15 seconds, then the response of the cell was observed in the microscopic field. The signal was recorded visually or continuously with a photoconductive cell coupled to a Hitachi video monitor focused on the microscopic field. The signal was recorded on a Health-Tec HT-100 data receiver (Omaha, Nebraska). The agonist concentration in the pipette ranged from 10⁻¹⁵ M to 10⁻⁴ M; the exact drug concentration after dilution in the chamber was not measured. The drug vehicle was also applied to exclude application artifact. Only one drug application per coverslip was performed to avoid possible agonist-induced desensitization with repeated exposure. The dose-response curves to norepinephrine with and without adrenergic innervation were determined for isolated sinoatrial node cells and for isolated ventricular muscle cells. Ventricular muscle cells were also observed after incubation in norepinephrine to assess agonist-induced desensitization. Dose-response curves to isoproterenol were determined for ventricular muscle cells, with and without adrenergic innervation, to assess the possible effect of neuronal uptake on norepinephrine sensitivity. Similarly, the dose-response curves to acetylcholine were determined for isolated sinoatrial node cells, with and without cholinergic innervation, and isolated ventricular muscle cells, with and without cholinergic innervation. Threshold and maximal response were determined to norepinephrine and acetylcholine in ventricular cells after cholinergic or adrenergic innervation, respectively.

Statistical Analysis

Weighted least-squares regression analysis was used to describe the dose-response relations. The independent variable was log base 10 of dose, and the dependent variable was percent change in contraction frequency. Polynomial models up to degree 3 (cubic term) were investigated. The analysis yielded 3 for each experiment the models of smallest degree that best described the dose-response relations.

Changes in chronotropic responsiveness were determined by calculating the mean drug concentration at 50% maximal responsiveness (ED₅₀) for the cells before innervation. This value was then compared with the mean drug concentration at which this same percentage change in contraction frequency was measured in cells after innervation. Significant differences in threshold and maximal change in contraction frequency were determined by Student's t test. For all analyses, a p<0.05 was accepted as significant.

Results

Morphology of Innervation to Sinoatrial Node Cells

The cell population of cultures prepared from sinus node regions and neuronal explants was heterogeneous and included the following: sinoatrial node cells, atrial muscle cells, fibroblasts of cardiac origin, neurons (either adrenergic or cholinergic), and connective tissue support cells of neural origin. At a density of 1×10⁵ cells/ml, each high power field (×320) contained three to four spontaneously contracting atrial muscle cells, while sinoatrial node cells were present in only every third to fourth high power field. The polyhedral atrial muscle cells were 50–90 μm in diameter and contracted vigorously at 85–135 contractions/min (mean 112±5, n=56). The
spindle-shaped sinoatrial muscle cells were 3–8 μm in diameter and 20–35 μm in length and had rapid, attenuated contractions at 95–300/min (185±8, n=42). Spontaneous contractions of both atrial muscle and sinoatrial node cells were uniformly present by 72 hours.

As the cardiac cells attached to the culture substrate and spontaneous contractions began, concurrent transformations occurred in the neuronal explants. Beginning 36 hours after preparation, individual neurons would migrate from both the sympathetic chain and the spinal cord explants to the dispersed cardiac cells (Figure 1A). Large radial projections also grew outward from the neural explants into the heterogeneous population of cardiac cells. The projections were bundles of usually two or more axons originating from neuron cell bodies remaining within the explants. The growth pattern of the explants over time failed to indicate the source of origin as either sympathetic chain or spinal cord.

All neurons were bipolar with spindle bodies (5–15 μm in size) and axons extending at either end with intermittent varicosities throughout their course. Terminal axons, whether from neurons remaining within the explants or from distally migrated neurons, extended distances ≤200 μm and established anatomical contact by 48 hours with both sinoatrial node and atrial muscle cells. These neuromuscular junctions were maintained despite the spontaneous contractions of the cardiac cells. The terminal axon movement was identical to that of the corresponding contracting cardiac cell. The neuromuscular junctions were varied in configuration. Axons might terminate after multiple arborizations, simply without arborization, or occasionally after widening into an apparent varicosity (Figure 1B). Both sinus node and atrial muscle cells were innervated. Neuromuscular junctions were uniformly established on the basis of proximity. Anatomic contacts between neurons were also established. At the light microscopic level, no definite morphological characteristic consistently distinguished between neurons from sympathetic or spinal cord origin. All neurons stained densely with Bodian's preparation.

Selective Innervation of Sinoatrial Node Cells

Neuronal maturation and identification were confirmed by indications of either selective adrenergic or cholinergic neurotransmitter synthesis. Glyoxylic acid and PAP preparations were made from all cultures after 4 days of incubation. Positive catecholamine histofluorescence was present only in cultures from sympathetic chain explants (Figure 2A). However, positive labeling with PAP was present only in cultures from sacrococcygeal spinal cord explants (Figure 2B). In the absence of explants, neurons could not be detected by either technique in the sinus node cultures. Thus, these two techniques confirmed the presence of solely adrenergic neurons in cultures with sympathetic explants and solely cholinergic neurons in cultures with spinal cord explants.

Selective neuroeffector transmission was confirmed by electrical stimulation of neurons (Figure 3). Stimulation of neurons from sympathetic chain explants produced only a positive chronotropic response. The chronotropic response was observed only in sinoatrial node cells on which the stimulated neuron terminated. Conversely, stimulation of neurons from sacrococcygeal spinal cord produced only a negative chronotropic response in sinoatrial node cells with anatomic contact with the stimulated neuron. These chronotropic responses to stimulation could be blocked by suffusion with 3×10⁻⁸ M propranolol or atropine sulfate, respectively, and thus confirmed selective adrenergic or cholinergic neurotransmission.

Innervated and noninnervated sinoatrial node cells were exposed to guanethidine sulfate or bethanecol chloride in the suffusion chamber. A positive chronotropic response to guanethidine was observed only in sinoatrial node cells innervated by sympathetic chain explants (n=8, 15.4±4%). In contrast, a negative chronotropic response was observed only in node cells innervated by sacrococcygeal spinal cord explants (n=7, 18.1±6%). Isolated sinoatrial node cells alone failed to respond to either guanethidine or bethanecol suffusion.

Thus, in every case, electrical or pharmacologic stimulation of neurons obtained from sympathetic chain explants demonstrated neurotransmission and the expected adrenergic response. Likewise, neurons from sacrococcygeal spinal cord exhibited neurotransmission and the expected cholinergic response following electrical or pharmacologic stimulation. Since these responses were identical to those observed in ventricular muscle cells,15 we chose not to stimulate every cell prior to neurotransmitter exposure.

Chronotropic Responses

The isolated sinoatrial node cells were supersensitive to both norepinephrine and acetylcholine. The response of the cell to each neurotransmitter exposure occurred almost instantaneously (Figures 4 and 5). The response threshold to norepinephrine was 3×10⁻¹⁰ M and to acetylcholine was 6×10⁻¹⁵ M (Figures 4, top, and 5, top, Table 1). For comparison, isolated ventricular muscle cells free of innervation were also exposed to norepinephrine and acetylcholine (Figures 4, bottom, and 5, bottom). Although highly sensitive, the isolated ventricular muscle cells were significantly less sensitive to the respective neurotransmitters than to the sinoatrial node cells: norepinephrine threshold 3×10⁻¹¹ M, p<0.05, acetylcholine threshold 6×10⁻¹⁴ M, p<0.05 (Table 2).

Following adrenergic innervation, the sinoatrial node cells remained supersensitive to norepinephrine (Figure 4, top). Although the dose-response curve was not identical to that of isolated sinoatrial...
FIGURE 1. Light photomicrographs of innervated sinoatrial node cells in primary culture. A: Migration of individual neurons out of sympathetic explants into dispersed sinoatrial and atrial cells. Axons crisscross one another and all other cells in culture to form lacy network. Phase contrast optics (original magnification, ×320). B: Axons that migrated from sacrococcygeal spinal cord explant have terminal bifurcations and have formed anatomic contacts on sinoatrial node cells. Axonal varicosities appear along the entire length of the axon. Silver stain (original magnification, ×600).
FIGURE 2. Demonstration of selective neurotransmitter synthesis. A: Positive glyoxylic acid histofluorescence of catecholamines in a sympathetic explant at 72 hours. The explant body, strongly fluorescent at top, has an axon bundle projecting from it containing multiple axons with fluorescent varicosities. Only sympathetic neuromuscular cultures contained positive fluorescence (original magnification, ×320). B: Positive peroxidase–antiperoxidase labeling of choline acetyltransferase within a neuron originating from sacrococcygeal spinal cord explants. Adjacent myocardial cell is unstained (original magnification, ×600).
**Atkins and Marvin** SA Node and Ventricular Cells After Innervation

**FIGURE 3.** Video recording of spontaneous contractions of single sinoatrial node cells following functional adrenergic and cholinergic neurotransmission. Arrows indicate point of microelectrode stimulation (frequency 5 Hz) performed with a toggle switch (on-off). Recording speed was 25 mm/sec, indicated by a 1-second time bar. A: Decrease in frequency from 180/min to 100/min after cholinergic neuron stimulation. This response was blocked by atropine sulfate suffusion $10^{-5}$ M, for 2 minutes. This experiment was repeated in three separate cultures, and only a negative chronotropic response was elicited by stimulation. B: Increase in frequency from 90/min to 120/min after microelectrode stimulation of an adrenergic neuron. This response could be blocked by $3 \times 10^{-5}$ M propranolol suffusion for 2 minutes. Experiments performed in three separate cultures produced similar positive chronotropic results.

**FIGURE 4.** Dose-response curves of sinoatrial node and ventricular muscle cells to norepinephrine with (○) and without (*) adrenergic innervation. Drug concentration is that in the pipette. The number of cells for each point is given in parentheses. Data displayed as mean±SEM. Top: Sinoatrial node cells. Mean control contraction frequency 185±8/min. Threshold of isolated cells was $3 \times 10^{-16}$ M. Mean contraction frequency of innervated cells was 179±11/min. Innervated cells had a threshold of $3 \times 10^{-14}$ M ($p<0.05$), but maximal response was unchanged. Bottom: Ventricular muscle cells. Mean contraction frequency of control myocytes was 93±6/min. Mean contraction frequency of innervated myocytes was 94±7/min. Isolated ventricular myocytes were less sensitive than sinoatrial cells, threshold being $3 \times 10^{-11}$ M. Unlike sinoatrial node cells, ventricular muscle cell response was altered after adrenergic innervation. The threshold was shifted rightward, and maximal response shifted downward.
node cells, the difference is primarily a change in threshold. Chronotropic responses at higher agonist concentrations were identical, which is a markedly different response than that of the ventricular cells. The maximal response was similar \((p>0.05)\). Following cholinergic innervation, the sinoatrial node cells remained supersensitive to acetylcholine; the dose response was virtually identical without a significant change in either threshold or maximal response (Table 1).

In contrast, the chronotropic responses of ventricular muscle cells were significantly altered by both adrenergic and cholinergic innervation. Following adrenergic innervation ventricular muscle cells had a significantly higher threshold \((3 \times 10^{-11} \text{ M} \text{ to } 3 \times 10^{-9} \text{ M}, p<0.05)\). The dose-response relation differed in both shape and degree of response \((p<0.05)\). Innervated cells demonstrated a smaller percentage increase in contraction frequency than the isolated cells (Figure 4, bottom). Maximal chronotropic response was decreased, while dose concentration at 50% maximal control response was increased (Table 2). These chronotropic changes indicated a desensitization of the ventricular muscle cells as a result of adrenergic innervation. To exclude neuronal uptake of norepinephrine as a plausible explanation, dose-response curves were determined using isoproterenol as the agonist (Figure 6, Table 2). Again, the response to isoproterenol revealed a threshold increase and a significant difference in the dose-response relation \((p<0.05)\). The response to isoproterenol of innervated myocytes was less at any given dose, and the group differences were larger at higher agonist concentration, indicating such desensitization occurred without

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**TABLE 1. Chronotropic Responsiveness of Sinoatrial Node Cells With and Without Autonomic Innervation**

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Isolated cells</th>
<th>After innervation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norepinephrine</td>
<td>Threshold</td>
<td>(3 \times 10^{-16} \text{ M})</td>
</tr>
<tr>
<td></td>
<td>Maximal increase in contraction frequency*</td>
<td>39.0±6%</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>Threshold</td>
<td>(6 \times 10^{-15} \text{ M})</td>
</tr>
<tr>
<td></td>
<td>Maximal decrease in contraction frequency*</td>
<td>40.7±5%</td>
</tr>
</tbody>
</table>

*Mean±SEM
†\(p<0.05\) compared with isolated cells
neuronal uptake. Although each cell was exposed only once to the neurotransmitter by application, the spontaneous release of norepinephrine from adrenergic neurons might also produce agonist-induced desensitization to account for these data. Ventricular muscle cells in the absence of neurons were incubated with norepinephrine $3 \times 10^{-8}$ M, replenished every 8 hours in the culture media. After 72 hours of incubation in norepinephrine, the dose-response curve (Figure 7) of such incubated cells to norepinephrine revealed a constant response threshold at $3 \times 10^{-11}$ M as with control cells but different from innervated cells. The decreased chronotropic responsiveness of ventricular muscle cells following selective adren-
cardial cell membrane. This culture system satisfies the initial onset of neurotransmission to the myocardium requires intact animals in whom major alterations in the sequential development of innervation may be produced. More advantageous is a system in which experiments are conducted with the initial onset of neurotransmission to the myocardial cell membrane. This culture system satisfies this requirement. The myocardial cells are harvested before in vivo neurotransmission but after in vivo establishment of anatomic contact and synthesis of neurotransmitter. Additionally, the in vitro neurons retain functional specificity during the experimental time course. Others have reported the ability of cultured sympathetic rat neurons to synthesize and accumulate acetylcholine during the second through fourth weeks of in vitro maintenance. Our experiments were conducted much earlier, on days 3 to 5, and although low levels of choline acetyltransferase have been detected, only adrenergic neuroeffector transmission has been demonstrated.

The spinal cord tissue contains presumably preganglionic parasympathetic neurons and spinal motor neurons, not the postganglionic neurons that innervate the heart. This constraint on our culture system is necessary since postganglionic cardiac neurons do not survive enzyme dispersion and, as of yet, are not readily dissected. Differences in neuromuscular response, cotransmitters, and tonic activity between postganglionic cholinergic cardiac neurons and those used in this study may confound the interpretation of the postjunctional response of myocardial cells. However, the response of the myocardial cells to neuronal stimulation was a muscarinic response, and thus, the use of sacrococcygeal spinal cord neurons to simulate the action of postganglionic nerves was accepted for this study.

Although it is not unreasonable to assume that autonomic innervation alters sensitivity of neurotransmitters, other prejunctional and postjunctional factors also potentially modulate the end organ response. Since the chronotropic responses to acetylcholine occurred almost instantaneously with pulsed drug application, alterations in acetylcholinesterase activity seem unlikely to explain the decreased muscarinic chronotropic response. Neuronal uptake of neurotransmitter cannot solely explain these data, since the response to isoproterenol was also reduced after adrenergic innervation. Decreased sensitivity in response to short- and

Discussion

These experiments confirm selective in vitro adrenergic or cholinergic innervation of cultured single sinoatrial node cells. Isolated sinoatrial and ventricular cells were chronotropically responsive to norepinephrine and acetylcholine at strikingly low drug concentrations, with isolated sinus node cells being more sensitive. Isolated sinus node cells displayed a blunted dose-response curve to both neurotransmitters that was generally unchanged after either adrenergic or cholinergic innervation. Ventricular cells, in contrast, demonstrated diminished sensitivity to norepinephrine and acetylcholine after adrenergic and cholinergic innervation, respectively.

Developmental changes in myocardial autonomic responsiveness occur concurrently with the onset of neuroeffector transmission, suggesting that the two events may be causally related. Confirmation of this theory, however, has been hampered by lack of a suitable experimental model. Chemical destruction of neurons or premature synapse formation requires intact animals in whom major alterations in the sequential development of innervation may be produced. More advantageous is a system in which experiments are conducted with the initial onset of neurotransmission to the myocardial cell membrane. This culture system satisfies the initial onset of neurotransmission but after in vivo establishment of anatomic contact and synthesis of neurotransmitter. Additionally, the in vitro neurons retain functional specificity during the experimental time course. Others have reported the ability of cultured sympathetic rat neurons to synthesize and accumulate acetylcholine during the second through fourth weeks of in vitro maintenance. Our experiments were conducted much earlier, on days 3 to 5, and although low levels of choline acetyltransferase have been detected, only adrenergic neuroeffector transmission has been demonstrated.

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![Figure 7. Dose-response curves of innervated ventricular muscle cells to norepinephrine (○) and isolated cells incubated in 3×10⁻³ M norepinephrine (•). Mean control frequency of cells incubated in norepinephrine was 89±5/min. Drug concentration is that in the pipette. Data displayed as mean±SEM. The number of cells for each point is given in parentheses.](http://circres.ahajournals.org/content/64/6/1060/F4.large.jpg)
long-term agonist exposure, agonist-induced desensitization, has been observed in multiple in vitro and in vivo systems. Incubation of the ventricular cells with norepinephrine produced sensitivity intermediate between that of isolated cells and cells after adrenergic innervation. Even though the concentration of norepinephrine in the growth medium was well into the range of high responsiveness, the responses of ventricular cells incubated in norepinephrine and those of innervated cells were not identical. Thus, agonist-induced desensitization mediated by endogenous release of neurotransmitter also is unlikely.

The sensitivity of sinus node cells to norepinephrine is several times greater than that reported elsewhere. Those studies employed either intact tissue preparations or disruption of the membranes for binding studies. In the former, the drug must diffuse through extracellular matrix and multiple cell layers to elicit a response; while in the latter, binding may be altered by the preparation of the membrane fragments. In this study, there was no diffusion through multiple cells, as the cells were isolated and the cell surfaces were in direct contact with the perfusing solution. These advantages may in part account for the marked increased sensitivity observed.

Of note was the differential response between the sinoatrial node cells and ventricular cells to both adrenergic and cholinergic innervation. The supersensitivity and lack of desensitization of the sinus node should maintain a chronotropic steady state during innervation and when the newborn may be particularly vulnerable to changes in heart rate, explaining disparate results from several laboratories. Löffelholz and Pappano examined the response of the isolated chick sinus node region to autonomic agents throughout the ontogenesis of both adrenergic and cholinergic innervation and failed to demonstrate a persistent alteration in sensitivity after the onset of innervation. In another study, Lau et al. were unable to evoke any changes in newborn rat heart rates to isoproterenol after denervation with 6-hydroxydopamine. They concluded that chronotropic responsiveness of the neonatal myocardium is not transsynaptically regulated. These results concur with the lack of desensitization observed in the sinoatrial cultures after adrenergic or cholinergic innervation. These results, however, fail to exclude the possibility of ventricular desensitization observed in this study, since the former examined only sinus node regions and, in the latter, the sinus node pacemaker dominance should override any ventricular changes.

In conclusion, we have demonstrated the effects of selective adrenergic and cholinergic innervation on chronotropic responsiveness in cultured isolated sinoatrial and ventricular muscle cells. Our data demonstrate that the addition of adrenergic or cholinergic neurons results in desensitization of ventricular but not sinus node cells. Future comparisons directed at specific myocardial cells should increase understanding of neurotrophic modulation, and this increased understanding may have implications in both normal and abnormal cardiac development.

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