**Neuronal Regulation of the Development of the α-Adrenergic Chronotropic Response in the Rat Heart**

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**SUMMARY.** During development, there are changes in the response of automatic cardiac fibers to α-adrenergic agonists. In neonatal rat ventricle, in vitro phenylephrine ($1 \times 10^{-6}$ M) induces an increase in automatic rate from $115 \pm 12$ (mean ± SEM) to $168 \pm 10$ beats/min, $P < 0.05$. In contrast, in adult rat ventricle, the rate decreases from $36 \pm 8$ to $12 \pm 12$ beats/min, $P < 0.05$. At both ages, the response is attenuated by the α-antagonist, prazosin ($1 \times 10^{-6}$ M). We used cultures of neonatal rat myocytes to determine whether maturation of innervation contributes to the ontogeny of this response. All non-innervated cultures showed a positive chronotropic response to α-stimulation; phenylephrine ($1 \times 10^{-6}$ M) increased the rate from $40 \pm 2$ to $52 \pm 2$ beats/min, $P < 0.05$. In contrast, 60% of the myocytes innervated with sympathetic neurons showed a decrease in rate in response to phenylephrine, from $78 \pm 6$ to $67 \pm 6$ beats/min, $P < 0.05$. The negative chronotropic effect of phenylephrine did not result from the release of acetylcholine or adenosine, or the inhibition of presynaptic norepinephrine release by phenylephrine. Furthermore, exposure to neuronal norepinephrine is not responsible for the alteration in muscle cell responsiveness. In conclusion, we have demonstrated the modulation of the myocardial response to α-adrenergic stimulation by the occurrence of innervation in tissue culture. This provides an explanation for the previously identified ontogenetic change in α-adrenergic effects on intact cardiac fibers from excitation to inhibition. *(Circ Res 57: 415-423, 1985)*
monitored with Teflon-coated bipolar silver wire electrodes connected to an amplifier. The signal was recorded on a strip chart recorder. The tissue was allowed to beat spontaneously in the bath for 20 minutes. Those preparations that attained a regular and stable rhythm within this time were monitored for stability for an additional 20 minutes, and then the experiment was begun. Each preparation was exposed to increasing concentrations of the \( \alpha \)-agonist, phenylephrine (1 \( \times \) 10\(^{-10} \) M to 1 \( \times \) 10\(^{-4} \) M). Steady state effects occurred in 5–8 minutes; the period of perfusion used with each concentration was 10 minutes. In experiments involving \( \alpha \)-blockade, prazosin (1 \( \times \) 10\(^{-8} \) M) was added to the bath after the control period. Perfusion with prazosin, alone, was maintained for 20 minutes before phenylephrine exposure and then was continued, with phenylephrine, throughout the experiment. This concentration of prazosin did not alter the spontaneous rate of beating. After exposure to the highest concentration of phenylephrine, a wash in Tyrode’s was performed, and the automatic rate was again determined.

Adult rats weighing 220–400 g were anesthetized with sodium pentobarbital administered intraperitoneally. The chest and abdominal cavities were opened, and the organs were removed, placed in sterile salt solution, and dissected free from associated tissue. The heart was then trimmed away. The trypsin dispersion was exposed to increasing concentrations of the \( \alpha \)-agonist, phenylephrine (1 \( \times \) 10\(^{-10} \) M to 1 \( \times \) 10\(^{-4} \) M) for 2 minutes at each concentration. The spontaneous rate changes due to the pipetting action of drug administration were monitored with Teflon-coated bipolar silver wire electrodes for 20 minutes. On discontinuation of stimulation, they attained a stable spontaneous rate and rhythm. The remainder of the protocol for the adult hearts was identical to that for the neonates.

**Preparation of Muscle Cell Cultures**

Under sterile conditions, hearts were removed from 2-day-old Wistar rats and placed in sterile salt solution. The atria were then trimmed away. The trypsin dispersion protocol has been described previously (Lau et al., 1980). Subsequently, the cells were pooled and centrifuged at 200 \( g \) for 5 minutes and were resuspended in Dulbecco’s minimal essential medium (MEM) with 10% horse serum, 5 \( \times \) 10\(^{-4} \) M hypoxanthine, and 12 mM NaHCO\(_3\). The cells were preplated for 40 minutes at 37°C to decrease fibroblast contamination (Blondel et al., 1971). The muscle cells then were resuspended in MEM containing 20 ng/ml nerve growth factor (NGF) at a density of 500,000 cells/ml (250,000 cells/mm\(^2\)) and plated in 60-mm petri dishes containing 5 \( \times \) 22 mm coverslips, each precoated with fibronectin (25 ng/mm\(^2\)), for 45 minutes.

**Preparation of Nerves for Nerve-Muscle Cultures**

After removal of the hearts from 2-day-old Wistar rats, the rest of the contents of the chest and abdominal cavities was removed. Then, the paravertebral sympathetic chains were removed, placed in sterile salt solution, and dissected free from associated tissue. The chains were incubated at 37°C in 0.125% trypsin for 30 minutes. After this period, MEM with 10% horse serum was added to stop the trypsinization. The cells were triturated approximately 40 times and the supernatant was collected. Additional trypsin was added to the remaining tissue, which was then triturated another 20 times and finally diluted with MEM and horse serum. The supernatants from the two triturations were pooled and centrifuged at 200 \( g \) for 5 minutes.
Materials
Sources: L-Phenylephrine hydrochloride, atropine sulfate, trypsin III, adenosine deaminase II, and fibronectin (Sigma Chemicals); prazosin (Pfizer); dl-propranolol (Ayerst); MEM with Hanks' salt solution (GIBCO); horse serum (KC Biological). NGF was provided by Dr. D.I. Ishii.

Statistics
All groups studied were tested for normal distributions of spontaneous rates with the D'Agostino test (Zar, 1974). All concentration-response curves were analyzed by nested analysis of variance (Snedecor and Cochran, 1967), and significant differences were determined at the P < 0.05 level. Differences in mean values at the P < 0.05 level were determined using the Bonferroni critical value for the modified t-test. The paired t-test (Zar, 1974) was used to determine differences between control rate and the effect of atropine or propranolol on the cultures. All values are expressed as mean ± SEM.

Results
Effects of Phenylephrine and Prazosin on Intact Tissue Automaticity
Adult Rat
We first asked whether the negative chronotropic effect of α-adrenergic stimulation previously observed in canine Purkinje fibers (Rosen et al., 1977; Posner et al., 1976) also occurred in adult rat ventricle. Phenylephrine induced a concentration-dependent decrease in rate in adult muscle, as shown in Figure 1A. The maximum negative chronotropic effect occurred at a concentration of $1 \times 10^{-8} \text{M}$, at which the spontaneous rate was $13 \pm 4$ beats/min, compared to the control rate of $36 \pm 8$ beats/min. In the presence of prazosin ($1 \times 10^{-6} \text{M}$), no concentration of phenylephrine significantly decreased the rate from the control value ($39 \pm 7$ beats/min). To confirm that the increase in rate induced by phenylephrine ($1 \times 10^{-6} \text{M}$) in the presence of prazosin was β-mediated, we conducted an additional protocol in the six hearts treated with prazosin. After completing the concentration-response curve, in the continued presence of prazosin, we first washed out the phenylephrine ($98 \pm 17$ beats/min) and then added phenylephrine ($1 \times 10^{-6} \text{M}$) and propranolol ($1 \times 10^{-7} \text{M}$), simultaneously. The rate no longer increased ($40 \pm 11$ beats/min). Finally, the phenylephrine and propranolol again were washed out ($39 \pm 11$ beats/min) and phenylephrine ($1 \times 10^{-5}$ alone was added. This increased the rate to $46 \pm 13$ beats/min, $P < 0.05$, demonstrating that the tissue was still capable of responding to phenylephrine after the extended drug regimen.

Neonatal Rat
In contrast to the adult rat ventricle, the neonatal ventricle exhibited a positive chronotropic response to all concentrations of phenylephrine. At a concentration of $1 \times 10^{-8} \text{M}$, the rate increased to $168 \pm 11$ from a control rate of $115 \pm 12$ beats/min (Fig. 1B).
TABLE 1
Catecholamine Levels in Nerve-Muscle Cultures and Muscle Cultures as a Function of Culture Age

<table>
<thead>
<tr>
<th>Catecholamine level (pg/ml)</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Muscle</td>
<td>Nerve-muscle</td>
<td>Muscle</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>16 ± 5</td>
<td>57 ± 4</td>
<td>25 ± 8</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>31 ± 9</td>
<td>9 ± 4</td>
<td>27 ± 9</td>
</tr>
<tr>
<td>Dopamine</td>
<td>38 ± 15</td>
<td>12 ± 10</td>
<td>19 ± 14†</td>
</tr>
</tbody>
</table>

n = 5 in each case (mean ± SEM).
* P < 0.05 compared to corresponding muscle cultures.
† P < 0.05 compared to catecholamine level on day 2.

cultures, the most dramatic difference was observed in the norepinephrine levels. Nerve-muscle cultures exhibited an increase from 57 ± 4 pg/ml on day 2 to 210 ± 40 pg/ml on day 5. In contrast to the nerve-muscle cultures, norepinephrine levels did not exceed 25 ± 8 pg/ml during the first 5 days in pure muscle cultures. Epinephrine and dopamine also exhibited a time-dependent increase in the nerve-muscle cultures (Table 1). The former remained low in the control muscle cultures, whereas the latter decreased significantly.

We next exposed day 4 and 5 cultures to tyramine (1 X 10^-6 M) to determine whether sufficient norepinephrine could be released to affect the spontaneous rate of the muscle cells in the nerve-muscle cultures. Figure 2 depicts a strip chart recording of the effect of tyramine on spontaneous rate in muscle and nerve-muscle cultures. The spontaneous rate of the muscle culture was not altered by tyramine. In contrast, the rate of the nerve-muscle culture increased from 69-92 beats/min. Although the magnitude of the increase in rate varied from culture to culture, 53% of the 45 nerve-muscle cultures exposed to tyramine demonstrated an increase in rate, whereas there was no increase in rate in any of the 40 non-innervated muscle cultures tested.

Effect of Phenylephrine on Spontaneous Rate of Muscle and Nerve-Muscle Cultures

Having confirmed the presence of releasable catecholamines in day 4 and 5 nerve-muscle cultures, we next compared the response of nerve-muscle cultures and pure muscle cultures of this age to phenylephrine. Figure 4 demonstrates the effect of two concentrations: 1 X 10^-8 M, which had induced
the maximum negative chronotropic response in adult rat ventricle, and $1 \times 10^{-8}$ M, which had induced a $\beta$-mediated positive chronotropic response in the adult rat. In the muscle culture, rate increased from 60-78 beats/min in response to $1 \times 10^{-8}$ M phenylephrine; a concentration of $1 \times 10^{-6}$ M further increased the rate to 96 beats/min. In contrast, the nerve-muscle culture responded to $1 \times 10^{-8}$ M phenylephrine with a decrease in rate from 66-58 beats/min. Phenylephrine ($1 \times 10^{-6}$ M) increased the rate to above the control level, presumably due to $\beta$-receptor activation.

To confirm that the positive chronotropic response of non-innervated muscle cultures was $\alpha$-mediated, we performed phenylephrine concentration-response curves in the presence and absence of prazosin. Phenylephrine induced a concentration-dependent increase in spontaneous rate in the pure muscle cultures, whereas pre-exposure to prazosin ($1 \times 10^{-6}$ M) depressed the positive chronotropic effect (Fig. 5).

Additional studies of the cultures suggested that the nerve-muscle cultures possess both parasympathetic and sympathetic tone. The muscarinic blocker, atropine ($1 \times 10^{-7}$ M), increased the spontaneous rate from 54 ± 6 to 60 ± 6 beats/min ($n = 16, P < 0.05$); propranolol ($1 \times 10^{-7}$ M) decreased spontaneous rate from 60 ± 6 to 56 ± 8 beats/min ($n = 16, P < 0.05$). In contrast, pure muscle cultures showed no response to either antagonist. Therefore, to eliminate the possibility that phenylephrine was acting pre-synaptically in the nerve-muscle cultures to cause the release of acetylcholine (resulting in muscarinic slowing of the rate), the response of nerve-muscle cultures to phenylephrine was measured in the presence of atropine ($1 \times 10^{-7}$ M). Propranolol ($1 \times 10^{-7}$ M) also was added to eliminate the possibility that the negative chronotropic re-
response was due to a phenylephrine-induced decrease of spontaneous norepinephrine release from adrenergic neurons, which then might act at the myocardial β-adrenergic receptor.

Figure 6 demonstrates the different chronotropic responses of muscle and nerve-muscle cultures to phenylephrine (1 × 10⁻⁸ M), and also illustrates the effects of the added antagonists. All of the pure muscle cultures responded to phenylephrine with an increase in rate, both in the absence and presence of atropine and propranolol. In contrast, 60% of the nerve-muscle cultures showed a decrease in rate in response to phenylephrine in the presence of atropine alone, 66% of the nerve-muscle cultures showed a decrease in rate in response to phenylephrine (n = 16; data not shown). This eliminated muscarinic slowing as a possible mechanism. In the presence of both atropine and propranolol, 63% of the nerve-muscle cultures responded to phenylephrine (1 × 10⁻⁸ M) with a decrease in rate. A third neurotransmitter, adenosine or a phosphorylated derivative, which could have been responsible for the observed decrease in rate, was also eliminated as a possible mediator. In the presence of adenosine deaminase (10 U/ml), atropine, and propranolol, 75% of the nerve-muscle cultures showed a decrease in rate (n = 20; data not shown).

Besides highlighting the dramatic differences between the muscle and nerve-muscle cultures, Figure 6 suggested that responses of the innervated cultures consisted of two populations. Therefore, we divided the nerve-muscle cultures into two groups, based on whether or not they responded to phenylephrine (1 × 10⁻⁸ M). However, after the nerve-muscle cultures had been separated into two groups on the basis of whether they exhibited a negative chronotropic response to phenylephrine (1 × 10⁻⁸ M), each subpopulation was normally distributed.

Figure 7 shows complete concentration-response curves for the nerve-muscle chronotropic response to phenylephrine in the presence of atropine (1 × 10⁻⁷ M) and propranolol (1 × 10⁻⁷ M). As described in the preceding paragraph, the nerve-muscle cultures were divided into two groups based on whether or not they responded to phenylephrine (1 × 10⁻⁷ M). All of the muscle cultures exhibited increases in rate which persisted in the presence of atropine and propranolol.
development of cardiac autonomic responsiveness have relied on chemical destruction of neurons (Yamada et al., 1980) or premature formation of neuronal synapses (Lau and Slotkin, 1979) in the intact animal. Using cell culture, we were able to study the contribution of nerves in isolation from confounding factors in the intact animal.

The developmental immaturity and lack of functional cardiac innervation in the newborn rat has been well documented (Pappano, 1977; Standen, 1978; Lipp and Rudolph, 1982). We confirmed that neonatal rat myocardial cultures exhibit a positive chronotropic response to α-stimulation with phenylephrine, and that this is antagonized by the α-specific antagonist prazosin. Our studies confirm the presence of functional sympathetic neurons in the nerve-muscle cultures. Evidence for this is provided by the assays of catecholamine level, which increased considerably by day 4 in culture, and by the ability of tyramine (1 \times 10^{-8} \text{ M}) to elicit a change in spontaneous rate in day 4 nerve-muscle cultures. In contrast, pure muscle cultures fail to respond to tyramine or to show increases in catecholamine levels; dopamine levels decreased significantly by days 4 and 5. Further evidence of functional contact between nerve and muscle cells in these cultures has been demonstrated previously, both electrophysiologically and ultrastructurally (Robinson, 1985).

The addition of sympathetic neurons to neonatal myocytes in co-culture resulted in the development of a negative chronotropic response to phenylephrine, similar to that observed in intact ventricle from adult rat. Furthermore, we did not observe any example of a negative chronotropic response to phenylephrine in the more than 60 non-innervated muscle cultures studied.

The response to phenylephrine was studied in the presence of various antagonists to determine whether the negative chronotropy might be presynaptically mediated. It has been reported that adrenergic neurons may become partially cholinergic when cultured with nonneuronal cells, i.e., myocardial cells and fibroblasts (Furshpan et al.,

\times 10^{-8} \text{ M}) with a decrease in rate; 63% of the cultures responded to that concentration of phenylephrine with a decrease in rate, from 46 ± 3 to 35 ± 3 beats/min. The remaining 37% responded with an increase in rate from 46 ± 5 to 57 ± 6 beats/min. Nerve-muscle cultures exposed to increasing concentrations of phenylephrine in the presence of prazosin (1 \times 10^{-6} \text{ M}), atropine (1 \times 10^{-7} \text{ M}), and propranolol (1 \times 10^{-7} \text{ M}) showed no change in rate.

Since the norepinephrine concentration is significantly elevated in the medium of some nerve-muscle cultures, we considered the possibility that norepinephrine was the neurotrophic agent responsible for modifying the α-adrenergic response. We investigated the relationship between the norepinephrine levels in 18 nerve-muscle cultures and the change in rate from control in response to phenylephrine (1 \times 10^{-8} \text{ M}). Two methods were used to perform these experiments (Table 2). In nine experiments, we collected media on day 4 for the norepinephrine assay. Fresh media were added, and later that day pharmacological testing was performed. In this way, we were able to determine the cumulative norepinephrine levels to which the culture had been exposed by day 4, and relate this to the response to phenylephrine. To assess more accurately the level of norepinephrine at the time of the experiment in the other nine cultures, we removed the old media and added fresh media on day 4, as above, and then collected a sample of fresh media immediately before adding the phenylephrine. With neither method was there a statistical correlation (correlation coefficient of less than 0.01) between norepinephrine level and the α-adrenergic response (Table 2).

\[ \text{Change in rate in response to phenylephrine,} \ 1 \times 10^{-8} \text{ M} \ (\text{beats/min}) \]

**Discussion**

The similar time course for the ontogeny of functional innervation and the changes in cardiac autonomic responsiveness of the developing organism have suggested that the onset of innervation might mediate these changes (Pappano, 1977). Previous attempts to investigate the role of innervation in the...
Therefore, atropine was used to block postsynaptic muscarinic receptors in the event that acetylcholine was released presynaptically. Atropine (1 × 10^(-5) M) caused a small increase in rate in the nerve-muscle cultures, suggesting the presence of some cholinergic neurons. Propranolol also altered the rate in the nerve-muscle cultures, suggesting the occurrence of endogenous catecholamine release.

The rate of beating in non-innervated muscle cultures was not affected by atropine or propranolol. Nonetheless, we performed the phenylephrine concentration-response curves in the presence of atropine and propranolol for both the muscle and nerve-muscle cultures. A third possible transmitter that has been identified in similar cultures is adenosine or a phosphorylated derivative (Pottier et al., 1983). However, sympathetic neurons cultured with myocytes remain adrenergic for the first 5 days (Mains and Patterson, 1973; Johnson et al., 1976, 1980). Landis (1980) reported that, during the first 4 days in culture, about 50% of the sympathetic vesicles in each terminal have dense cores (characteristic of adrenergic neurons). The purinergic neurotransmitter was seen in cultures only after 2 weeks. Our studies were done on cells that had been in culture for 4–5 days. Therefore, it seemed unlikely that a purinergic neurotransmitter was responsible for the negative chronotropic response. Nevertheless, we thought it advisable to test the possibility of a purinergic mechanism and did this with adenosine deaminase. We demonstrated that the negative chronotropic response of nerve-muscle cultures to phenylephrine persisted in the presence of adenosine deaminase. Furthermore, both the negative and positive chronicotropic responses of nerve-muscle cultures to phenylephrine were prevented by the α₂-antagonist, prazosin, suggesting that any pre-synaptic action of phenylephrine would have to be via α₂-receptors, rather than the more likely α₁-subtype (Bertelssson and Pettaguer, 1977).

The reason that some of the nerve-muscle cultures persisted in showing an α-adrenergic increase in rate, rather than a decrease in rate, is unclear. It may depend on variations in nerve density, and, therefore, concentration of any conditioning agents in the bulk medium (see below). Alternatively, this increase may result from the following factors. The nerve cells were dispersed throughout the culture, sending out processes that terminated on some muscle cells (Fig. 3), but leaving other muscle cells apparently non-innervated. Although muscle cells selected for study appeared to be innervated, upon visual inspection, we could not be certain of the presence of functional synapses in all instances. Further, in any particular instance, we could not know the degree of apposition of any synapses formed with the actual pacemaker cell within a contiguous, synchronous sheet of automatic myocytes.

We have previously considered the cellular electrophysiological mechanisms responsible for α-adrenergic induced negative and positive chronotropic effects on canine Purkinje fibers (Rosen et al., 1977; Redder et al., 1984). To summarize the earlier studies, the addition of α-adrenergicamines induces either a decrease (for the negative chronotropic effect) or an increase (for the positive chronotropic effect) in the slope of phase 4 depolarization. No changes in maximum diastolic potential or threshold potential were seen. Posner et al. (1976) reported on the negative chronotropic effects of epinephrine, and correlated these with a decrease in K⁺ flux across the membrane. One report of α-adrenergic effects on pacemaker currents has been inconclusive (Hauswirth et al., 1976). Hence, it appears reasonable to relate the α-adrenergic effects on chronotropy to an alteration in phase 4 depolarization, in association with changes in K⁺ permeability of the membrane.

The mechanism by which the addition of sympathetic neurons to cultured myocardial cells changes the α-adrenergic response in most cells studied also remains to be determined. It is possible that the effect is chemically mediated. Norepinephrine, the level of which varies from culture to culture, has been reported to modify post synaptic β-receptors: chronic exposure to β-agonists causes supersensitivity (Hemmsmeyer and Robinson, 1977; Tse et al., 1979; Yamaguchi et al., 1981), whereas reduction of sympathetic input results in supersensitivity (Glaubiger et al., 1978; Yamada et al., 1980; Lau and Slotkin, 1982). To determine whether norepinephrine in the culture media modulated the α-receptor response to phenylephrine, we measured norepinephrine levels in the nerve-muscle cultures before testing them pharmacologically (Table 2). We found no correlation between the norepinephrine level in a particular nerve-muscle culture and the presence or magnitude of the chronotropic response to α-adrenergic stimulation. Other bulk factors, the levels of which may fluctuate among cultures, may also be responsible for modifying the α-chronotropic response. Variations in local release of a conditioning factor provide another possibility.

In conclusion, we have used a cell culture preparation to elucidate the direct effects of innervation on autonomic responsiveness in the ventricular myocardial cell. Our data demonstrate that the addition of sympathetic neurons to cultured neonatal myocytes converts the α-response from an increase in rate to a decrease in rate, indicating that the ontogeny of innervation in vivo contributes to the observed conversion of the α-adrenergic response from positive to negative chronotropy during development. These results have the following important implications: first, they demonstrate that, in the postnatal period, the development of innervation has, in addition to its previously demonstrated physiological effects, the ability to change the qualitative response to α-adrenergic receptor stimulation of the heart. Since sympathetic and parasympathetic stim-
ulation are important modulators of cardiac rate and rhythm, the type of change we describe (from excitatory to inhibitory) might have an important antiarrhythmic role. Moreover, the failure of such changes to occur in normal fashion (a variability in the aforementioned changes) may contribute to arrhythmogenesis. A second question raised by our results is whether—if innervation, once established, is removed—the response to α-adrenergic stimulation again becomes excitatory. Such an event would have important implications with respect to myocardial infarction, where nerve death can occur, and to cardiac transplantations.

References

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