In Vitro Adrenergic and Cholinergic Innervation of the Developing Rat Myocyte

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SUMMARY. We studied the development of selective adrenergic and cholinergic neuroeffector transmission in primary cultures of isolated ventricular muscle cells. Explants of either thoracolumbar sympathetic ganglia or sacrococcygeal spinal cord were added to newborn rat ventricular cultures harvested prior to the onset of in vivo autonomic innervation. Neuronal growth, migration, and the formation of neuromuscular junctions were observed with light and scanning electron microscopy. Glyoxylic acid histofluorescence, reflecting catecholamine synthesis, was found in only the sympathetic neuromuscular cultures. Choline acetyltransferase activity was detected in both spinal cord and sympathetic neuromuscular cultures, but was significantly higher in the spinal cord neuromuscular cultures. The isolated ventricular muscle cells remained at a constant spontaneous contraction frequency, regardless of the type of culture preparation. Guanethidine sulfate application produced a positive chronotropic response, blocked by propranolol, in the sympathetic neuromuscular cultures, but not in the spinal cord neuromuscular cultures. Bethanechol sulfate produced a negative chronotropic response, blocked by atropine, in the spinal cord neuromuscular cultures, but not in the sympathetic neuromuscular cultures. Isolated ventricular muscle cells in the absence of neurons failed to respond to either agent. Direct microelectrode stimulation of adrenergic or cholinergic neurons likewise respectively produced either a positive or negative ventricular muscle cell chronotropic response. These studies are the first to establish the selective production of functional cholinergic and adrenergic innervation of isolated cardiac muscle cells in vitro. (Circ Res 55: 49-58, 1984)

THE interdependence of skeletal muscle and motor neuron, during both embryonic development and the maintenance of the mature functional state, has undergone extensive reappraisal (Vrbova et al., 1978). New interpretations of prior knowledge have been stimulated by the preparation of neuromuscular cultures (Peterson and Crain, 1970). This experimental approach has been expanded to investigate neuromuscular dystrophy in both animals and humans (Dubowitz, 1977).

Our understanding of cardiac and autonomic nervous system interdependence has extended beyond classic organ system response to reflex regulation. Denervation experiments in intact animals or tissue preparations (Fleming et al., 1973) and recent human cardiac transplantation (Mackintosh et al., 1982) have established the role of innervation in maintaining the mature myocardial cell membrane. Neuromuscular interdependence during cardiovascular development has been suggested by descriptive reviews of cardiac autonomic ontogenesis (Pappano, 1975, 1977; Marvin et al., 1980). Various studies report that in the immature heart, developmental changes in action potentials, agonist sensitivity, binding sites, and ionic channels occur at the commencement of autonomic neurotransmission (Sperelakis, 1972; Löeffelholz and Pappano, 1974; Galper et al., 1977; Sperelakis and McLean, 1978).

To relate developmental changes causally in the dynamic myocardial cell membrane to neurotrophic influence, autonomic innervation requires experimental acceleration or deceleration (Pappano, 1977).

In recent autonomic culture preparations, plasticity by neurotransmitter function has been said to follow environmental alteration of neuronal maturation, e.g., simultaneous sympathetic and cholinergic activity from a single neuron or neuronal explant (Patterson, 1979; Landis, 1980; Weber, 1981; Black, 1982). This heterogeneity of neuronal activity could be expected to produce an inconsistent neurotrophic influence on cardiac muscle cells in neuromuscular cultures, interfering with experimental results. Assessment of neurotrophic alteration of the postsynaptic receptor and membrane conductance mechanism, whether cholinergic or adrenergic, would be facilitated by homogeneous neuroeffector transmission.
muscle cells, morphologically and functionally comparable to the in vivo source and highly sensitive to neurotransmitters (Hermsmeyer and Robinson, 1977; Marvin et al., 1979). This study was designed to establish adrenergic and cholinergic innervation selectively of isolated cardiac muscle cells within neuromuscular cultures. Neurons were added to primary cultures of ventricular cells harvested just prior to the onset of functional autonomic innervation of the rat heart in vivo. Neuromuscular junctions developed and were observed by light and scanning electron microscopy. Neurotransmitter synthesis was reflected by measurements of choline acetyltransferase and dopamine β-hydroxylase activities or catecholamine histofluorescence. Positive or negative chronotropic alteration of spontaneous contraction frequency, in response to stimulation of adrenergic or cholinergic neurons, demonstrated the establishment of respective neuroeffector transmission to the isolated contracting cardiac muscle cells.

Methods

Cell Cultures

All culture preparations utilized newborn rats taken from the breeding colony of Kyoto-Wistar normotensive rats maintained in the Cardiovascular Center at the University of Iowa. Under aseptic conditions, ventricular apices, excluding all valvular and atrial tissue, were removed from 15–24 newborn rat hearts, minced with fine dissecting scissors into fragments approximately 1 mm in diameter, and placed in CV3M culture medium. The composition of the CV3M culture medium consisted of 85% MEM-Earle’s salts, 15% horse serum, 4 mM L-glutamine, 20 μg/ml gentamycin, 20 mM HEPES buffer (pH 7.3), and 16 mM NaHCO3. Following the completion of dissection, the ventricular fragments were dispersed with trypsin into a cell suspension as previously described (Marvin et al., 1979). Total dissection and trypsin dispersion time was approximately 3 hours.

Explants of neurons were prepared separately from, but simultaneously with, the primary cultures of ventricular cells. Under aseptic conditions and using the same newborn rats, the paravertebral thoracolumbar sympathetic chains and the sacrococcygeal spinal cord were removed from each animal, placed in CV3M culture medium, and minced into fragments approximately 0.5 mm in diameter. Separate instruments and dishes were utilized for the two neuronal preparations to prevent cross-neuropathic contamination. Three or four fragments from either neuronal source were subsequently plated on a 9 × 22 mm sterile glass coverslip in a single drop of CV3M culture medium. Two or three drops with the identical neuronal source were placed on a single coverslip. The coverslips were then incubated at 37°C in a 5% CO2 incubator for 2–3 hours to allow attachment of the neuronal explants to the coverslip surface.

While the neuronal explants were being incubated for attachment, trypsin dispersion of the ventricular tissue fragments was completed. The ventricular cell suspension, at a density of 10^6 cells/ml, was plated into petri dishes, each containing six glass coverslips. The coverslips in each petri dish, prior to ventricular cell plating, would either be totally empty or contain either sympathetic or sacral cord explants. These culture preparations then were incubated as described above.

These cultures were used for all experimental observations except for measurements of choline acetyltransferase and dopamine β-hydroxylase activity. All cultures prepared for these experiments were plated directly onto the petri dish surface, rather than on glass coverslips, to facilitate cell removal. The MEM-Earle’s salts, horse serum, L-glutamine, and trypsin were obtained from K & C Biological, Inc. The gentamycin was from Schering Corp., and the HEPES buffer was from Calbiochem.

Microscopic Observations

All cultures were observed using Leitz Diavert microscopes with either phase contrast or schlieren contrast optical systems. The glass coverslips containing cultured cells were observed in an open, thermally regulated suffusion chamber mounted on the microscopes. These suffusion chambers are described elsewhere (Hermsmeyer and Robinson, 1977). After observation of the living cells, the coverslips were removed for histofluorescent, histochemical, or scanning electron microscopic examination.

Preparation for catecholamine histofluorescence utilized a modified glyoxylic acid technique (Furness and Costa, 1975). The glass coverslips containing the cultures were rinsed rapidly in 0.15 M phosphate buffer (pH 7.6) at 37°C and transferred to a 2% solution of glyoxylic acid (Sigma) in phosphate buffer (pH 7.0) for 3 minutes at 25°C. The coverslips were then mounted on standard glass slides, dried in room air for 5 minutes, and heated in an oven 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. Bodian’s standard silver histochemical staining technique was also performed on cultures not prepared for histofluorescence examination (Luna, 1964). Cells, not examined by these microscopic methods, were observed under the scanning electron microscope, following fixation, dehydration, drying, and mounting as previously described (Marvin et al., 1979).

Enzyme Activity Determinations

After 96 hours of incubation, isolated ventricular cell cultures and neuromuscular cultures, of either sympathetic chain or spinal cord explants with cardiac cells, were collected separately for both dopamine β-hydroxylase and choline acetyltransferase assays. The radioenzymatic assay technique for dopamine β-hydroxylase activity has been reported (Coyle and Axelrod, 1972; Dickson et al., 1981). Choline acetyltransferase activity was assayed as previously described (Roskoski et al., 1974, 1975, 1977). Since we have previously reported that no choline acetyltransferase activity is detectable in primary ventricular cell cultures from newborn rats (Marvin et al., 1980), these determinations were not included in the design of this study.

Neuroeffector Transmission

Designated coverslips containing isolated ventricular cells or neuromuscular cultures were placed in the open suffusion chamber and allowed to equilibrate for 5–15 minutes. Suffusion, without recirculation at 37°C, was driven by a Gilson Mini Pulse pump. The O2-CO2 satu-
FIGURE 1. Scanning electron photomicrographs of neuronal explants at 72 hours of age in primary ventricular cell cultures. The topographic features of the two different neuromuscular culture preparations are indistinguishable. Both explants send large axonal projections into the peripheral ventricular cells. Panel A: paravertebral sympathetic explant in foreground with radial projections into background ventricular cells, 110X. Panel B: sacrococcygeal spinal cord explant again in foreground. The artefactual winding course of axonal projections was produced by explant detachment during fixation, 200X.

rated suffusion solution was isotonic and consisted of 130 mM NaCl, 16 mM NaHCO3, 0.5 mM NaH2PO4, 4.7 mM KCl, 1.8 mM CaCl2, 0.41 mM MgCl2, 0.41 mM MgSO4, 5.5 mM dextrose, and 13.0 mM HEPES (pH 7.6). Details of the suffusion chamber (200 µl) are given elsewhere (Hermsmeyer and Robinson, 1977).

Spontaneously contracting isolated ventricular muscle cells, with or without neuronal axons terminating on their surfaces, were observed, and the baseline contraction frequency recorded. Either 10 ng/ml guanethidine sulfate (Ciba) or bethanechol chloride (Merck, Sharp, and Dohme) were applied as a 10-µl pulse injection by Eppendorf pipette at the inlet of the suffusion chamber. During drug exposure, successive 15-second counting periods recorded the spontaneous contraction frequency. The peak frequency during drug exposure was compared to peak control frequency. In each case, the frequency returned to baseline values when drug exposure ended, and only one cell per coverslip was studied to avoid drug desensitization. The positive or negative chronotropic response was
considered adrenergic or cholinergic if preceding suffusion of 10 μg/ml propranolol or atropine (Sigma), respectively, would prevent the response.

Direct field stimulation of individual adrenergic or cholinergic neurons, whose axons terminated on an isolated ventricular muscle cell, was performed using a platinum microelectrode and a Grass 544 stimulator (1–5 mV, 2-msec duration, and 0.5-msec delay). The frequency of spontaneous contractions of the innervated ventricular muscle cells was measured continuously by a photosensitive cell coupled to a Hitachi video monitor of the microscopic field (Sinclair et al., 1970; Kupfer et al., 1982). The signal was recorded on a Health Tech HT-100 data transceiver. The cells were continuously suffused with the previously described O₂-CO₂ saturated isotonic solution. Stimulation artifact was excluded by microelectrode field stimulation of isolated ventricular muscle cell cultures without neuronal explants. As with the pharmacological stimulations, the chronotropic response was considered adrenergic or cholinergic if preceding suffusion of propranolol or atropine respectively prevented any frequency change.

Statistics

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

Results

Morphology of Neuromuscular Cultures

With the described culture methods, the light microscopic observations of both sympathetic chain and spinal cord explants and individual neurons were similar. Circumferential explant outgrowth began between 12 and 18 hours after attachment to the coverslip surface. Over the next 24–36 hours, large radial projections 10–25 μm in diameter, each containing several axons, extended 1–2 mm into the dispersed ventricular cells (Fig. 1). After 48 hours, many of the isolated ventricular muscle cells were spontaneously contracting, displacing, but not dislodging, the individual axons terminating on the muscle cell surfaces. The growth patterns and morphology of the two different explants were identical.

In addition to the axons originating from the explants, individual neurons by 72 hours had migrated from the explants distances up to 7 mm into the periphery (Fig. 2). These individual neurons were bipolar in configuration with central bodies up to 5 by 15 μm in size. From either pole of a central body, one or two axons could extend distances up to 200 μm to ventricular cells. Both the neuron bodies and axons stained densely with Bodian’s technique, as opposed to the ventricular cells. Varicosities were readily apparent throughout the axons. Bodian’s technique stained neurons in both sympathetic and spinal cord neuromuscular cultures.

The axons frequently terminated on the contracting muscle cells after passing over the surfaces of ventricular nonmuscle cells (Fig. 3). Less commonly, an axon would terminate on the body of a migrating neuron. Axons from several neurons would cross each other, forming a fine network in areas of high cell density. The crossing axons adhered to each other, hence displacement of a terminal axon on a contracting muscle cell would also oscillate the proximally crossing axonal network.

The configurations of the terminal axons observed were variable (Fig. 4). Many axons would divide into multiple ramifications proximal to the muscle cell surface. The endings of these ramifications, or of axons which did not divide, were inconsistent. Some would widen into a varicosity, others would
become smaller in caliber, and many appeared no different from the immediate proximal configuration. Fewer terminal varicosities and ramifications were seen in the spinal cord neuromuscular cultures compared to the sympathetic neuromuscular cultures. Regardless of the terminal configuration, the axons in both neuromuscular cultures were not dislodged by spontaneous contractions. Terminal configurations varied considerably on muscle cell surfaces despite origin from the same proximal neuron.

No neurons were found in the primary cultures of ventricular cells without added neuronal explants, either in the living state or after Bodian's stain. Any neurons seemingly present in the intact heart were destroyed by the trypsin dispersion.

Indications of Selective Neurotransmitter Synthesis

Glyoxylic acid preparations were made on all cultures at 4 days of incubation. Histofluorescence was consistently present in the neuromuscular cultures with sympathetic explants (Fig. 5). The histofluorescence was readily apparent in the neuronal bodies and the varicosities throughout the course of axons. Histofluorescence was not observed in any cultures of isolated ventricular muscle cells or in any
neuromuscular cultures with spinal cord explants. Controls for histofluorescence were positive whole mounts of adult rat kidney and spleen.

Radioassay of dopamine β-hydroxylase was likewise performed on all culture preparations at 96 hours of age. None of these determinations demonstrated any detectable dopamine β-hydroxylase activity in any of the culture preparations. Background activity of tissue aliquots failed to exceed the activity of blanks. The detection limit of the dopamine β-hydroxylase radioassay was 100 nmol/hr per mg protein. Choline acetyltransferase activity was determined only in the neuromuscular cultures. Choline acetyltransferase activity was detectable in both neuromuscular cultures, but was significantly higher in the cultures with the spinal cord explants, 7.3 ± 1.3 pmol/min per mg protein compared to 3.5 ± 1.8 pmol/min per mg protein in the sympathetic neuromuscular cultures. The two enzyme activity determinations were made on aliquots from 42 primary ventricular cell cultures, 30 sympathetic explant neuromuscular cultures, and 27 spinal cord neuromuscular cultures.

Table 1 summarizes the experiments assessing neurotransmitter synthesis. Only acetylcholine could be expected to be synthesized by the neurons remaining in the spinal cord neuromuscular cultures. Both norepinephrine and acetylcholine synthesis...
FIGURE 5. Positive glyoxylic acid histofluorescence of catecholamines. These were typical neurons in sympathetic neuromuscular cultures at 96 hours of age. The histofluorescence strikingly outlined the neuron bodies, axons, and axonal varicosities. Such histofluorescence was present only in sympathetic neuromuscular cultures.

were indicated in the sympathetic neuromuscular cultures. No evidence for either adrenergic or cholinergic neurotransmitter synthesis was found in the primary ventricular cell cultures alone.

**Development of Neuroeffector Transmission**

The onset of neuroeffector transmission was determined by direct pharmacological stimulation of the various culture preparations (Table 2). The pulsed application of 10 μl of guanethidine sulfate, 10 ng/ml, consistently produced a positive chronotropic response (mean = + 28.8 ± 2.4%) in the spontaneous contractions of isolated ventricular muscle cells with axons, from either sympathetic chain explants or single neurons, terminating on their surface membranes. Spontaneously contracting

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Observations of Neuronal Maturation in Spontaneously Contracting Ventricular Muscle Cell Cultures, with and without Neuronal Explants</th>
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<tbody>
<tr>
<td>Glyoxylic acid histofluorescence</td>
<td>Dopamine β-hydroxylase activity (pmol/min per mg protein)</td>
</tr>
<tr>
<td>Ventricular muscle cells with sympathetic explants</td>
<td>++++</td>
</tr>
<tr>
<td>Ventricular muscle cells with sacral cord explants</td>
<td>0</td>
</tr>
<tr>
<td>Ventricular muscle cells without explants</td>
<td>0</td>
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* Detection limit = 100 nmol/hr/mg protein.
† Marvin et al., 1980.
TABLE 2
Onset of Selective Adrenergic or Cholinergic Neuroeffector Transmission in Primary Cell Cultures of Contracting Ventricular Muscle Cells

<table>
<thead>
<tr>
<th></th>
<th>Baseline contraction frequency (contractions/min)</th>
<th>Rate increase with 10 ng/ml guanethidine sulfate*</th>
<th>Rate decrease with 10 ng/ml bethanechol chloride†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventricular muscle</td>
<td>88.7 ± 1.6 (n = 82)</td>
<td>28.8 ± 2.4%* (n = 14)</td>
<td>No response</td>
</tr>
<tr>
<td>cells with sympathetic explants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ventricular muscle</td>
<td>96.1 ± 4.5 (n = 59)</td>
<td>No response</td>
<td>30.0 ± 4.4%† (n = 13)</td>
</tr>
<tr>
<td>cells with sacral</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>cord explants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ventricular muscle</td>
<td>94.2 ± 3.3 (n = 91)</td>
<td>No response</td>
<td>No response</td>
</tr>
<tr>
<td>cells without explants</td>
<td></td>
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* Blocked by addition of 1.0 µg/ml propranolol.
† Blocked by addition of 1.0 ng/ml atropine.

ventricular muscle cells, in either the primary ventricular cultures alone or the neuromuscular cultures with spinal cord explants, failed to demonstrate any change in contraction frequency when guanethidine sulfate was applied, even with concentrations up to 1.0 µg/ml.

In contrast to these results, the pulsed application of 10 µl of bethanechol chloride consistently produced a decrease in the contraction frequency (mean = −30.0 ± 4.4%) of isolated ventricular muscle cells with axons from either spinal cord explants or single neurons terminating on their surface membranes. No change in contraction frequency was observed in the cultures of isolated ventricular muscle cells alone, or with the added sympathetic explants.

There was no significant difference in the mean contraction frequency of the ventricular muscle cells in any of the culture preparations prior to the application of either drug.

The failure of isolated ventricular muscle cells alone to respond to drug application, again documenting the inability of neurons to survive the trypsin dispersion, indicated the responses seen in the neuromuscular cultures were a direct result of neurotransmitter release from the neurons. Guanethidine sulfate displaced norepinephrine from the sympathetic neurons. The negative result to guanethidine sulfate in the spinal cord neuromuscular cultures was consistent with the absence of histofluorescence in these cultures. Bethanechol chloride application resulted in acetylcholine release from the spinal cord neurons. The failure of bethanechol chloride to result in any chronotropic change in sympathetic neuromuscular cultures, despite the presence of low choline acetyltransferase activity, indicated too little acetylcholine was released to stimulate the ventricular muscle cell, either directly (decreased contraction frequency) or indirectly through an interneuronal synapse (increased contraction frequency). Therefore, selective neuroeffector transmission, either adrenergic or cholinergic, was confirmed by these pharmacological experiments.

Selective neuroeffector transmission was also confirmed by microelectrode stimulation of adrenergic and cholinergic neurons (Fig. 6). Stimulation of adrenergic neurons, with axons terminating ventricular muscle cells, consistently produced a positive chronotropic response, mean frequency increase of 130 ± 12% (n = 11). Stimulation of cholinergic neurons terminating on muscle cell surfaces conversely resulted in a negative chronotropic response, mean frequency decrease of 45 ± 6.5% (n = 10). Prior to 72 hours of culture age and, occasionally, in older cultures, neuronal stimulation failed to produce any chronotropic change with both pharmacological and microelectrode stimulation. Thus, mere anatomical proximity of axons to muscle cells does not indicate

Figure 6. Video recordings of spontaneous contractions of single ventricular muscle cells following functional adrenergic and cholinergic neurotransmission. Arrows indicate point of microelectrode stimulation of neurons. Recording speed was 25 mm/sec, indicated by 1-second time bar. Panel A: increase in frequency from 47/min to 115/min followed adrenergic neuron stimulation by microelectrode. This response was blocked by 10^{-8} g/ml propranolol suffusion for 2 minutes. Panel B: decrease in frequency from 88/min to 54/min followed cholinergic neuron stimulation by microelectrode. This response was blocked by 10^{-8} g/ml atropine suffusion for 2 minutes.
either neuronal maturation or the presence of functional neuroeffector transmission.

Discussion

These experiments demonstrate the development of functional autonomic innervation of isolated cardiac muscle cells in vitro. The sequence of innervation occurred in a pattern similar to in vivo ontogenesis (Löffelholz and Pappano, 1974; Marvin et al., 1980), i.e., the establishment of anatomical neuromuscular contact, the evolution of neurotransmitter synthesis, and the subsequent functional neuroeffector transmission resulting in a postjunctional response. We believe these cell culture data have demonstrated the cellular nature of selective adrenergic and cholinergic neuroeffector transmission. This in vitro model indicates that cellular function is specified within the source organ, giving features with similar morphological and pharmacological characteristics at the cell level.

Denervation experiments have certainly provided useful insight into the maintenance of the mature functional muscle cell state (Guth, 1968; Gutmann, 1969; Fleming et al., 1973); however, a denervated mature muscle cell cannot be equated with an immature myocyte which has never been exposed to neurotrophic influence. Primary cell culture of rat ventricular cells at birth provides a practical opportunity to harvest such immature myocytes. Although autonomic neurons are present in the rat heart during embryonic cardiac organogenesis, functional innervation does not develop until after birth. Earliest cholinergic neuroeffector transmission in the rat heart can be detected consistently one day after birth, but the relatively mature levels of choline acetyltransferase activity in both atria and ventricles are not reached until between 7 and 14 days postgestation (Marvin et al., 1980). Development of mature adrenergic innervation in the rat heart is further delayed until 21 days postgestation (Pappano, 1975; Lais et al., 1976). Little, if any, significant neurotrophic modulation of cardiac development by either cholinergic or adrenergic neuroeffector transmission may have occurred at birth in the rat. Any neuronal alteration of these ventricular muscle cells before culturing would be limited to the trophic influence of extracellular secretions other than neurotransmitters.

Notwithstanding the importance of timing the interruption of cardiac development in vivo to prepare neuromuscular cultures, the time period selected to examine cellular events in vitro may be even more crucial to experimental interpretation. Aging cardiovascular muscle cells in culture may fail to maintain functional characteristics of the original in vivo source by undergoing the phenomenon of cell dedifferentiation (Chamley et al., 1977). The isolated ventricular muscle cells, maintained by the presented culture methods through the time interval of this study, retain phenotypic integrity as assessed by morphological and functional criteria (Marvin et al., 1979). Recently, investigators have demonstrated rat sympathetic neurons develop the ability to synthesize and accumulate acetylcholine and lose their ability to synthesize catecholamines in tissue cultures (Patterson and Chun, 1977; Patterson et al., 1978). This change in phenotypic expression, however, becomes manifest during the 2nd, 3rd, and 4th weeks of in vitro maintenance. We utilized the sympathetic neuromuscular cultures much earlier, from 72 to 96 hours, and although low levels of choline acetyltransferase activity were detected, only adrenergic neuroeffector transmission was present. Therefore, considering the appropriate time and culture conditions, both cardiac muscle cells and neurons in vitro will maintain functional expression, similar to that observed in vivo.

Our neuromuscular cultures have theoretical limitations. The cholinergic neurons from the spinal cord are for the most part preganglionic, and thus may not be totally representative of the cardiac postganglionic neurons. Unfortunately, the cardiac postganglionic neurons do not survive culture preparation (Roskoski et al., 1977). The ratio of neurons to cardiac muscle cells is artificial, and therefore the quantitative neurotrophic influence in neuromuscular cultures can be only qualitatively compared to cardiac modulation during the course of normal development. Biochemical assays cannot be expected to determine levels of activity comparable to that observed in vivo (Marvin et al., 1980), or to be sensitive enough to detect very small levels of activity at the cell level, e.g., the absence of dopamine β-hydroxylase activity in this study.

These limitations aside, we have demonstrated the selective adrenergic and cholinergic innervation of isolated cardiac muscle cells in vitro with functional neuroeffector transmission to single contracting cardiac muscle cells. Comparison of the electrophysiological and pharmacological characteristics of these cardiac muscle cells, after the onset of neuroeffector transmission, to isolated cardiac muscle cells, free of neurotrophic influence, may provide insight into the modulation of cardiac development by autonomic innervation. The in vitro neuronal retention of specific adrenergic and cholinergic cellular function may also allow studies on nerve-muscle interactions with dual innervation.

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