Phenotypic properties of adult mouse intrinsic cardiac neurons maintained in culture

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Hoard JL, Hoover DB, Wondergem R. Phenotypic properties of adult mouse intrinsic cardiac neurons maintained in culture. Am J Physiol Cell Physiol 293: C1875–C1883, 2007. First published October 3, 2007; doi:10.1152/ajpcell.00113.2007.—Intrinsic cardiac neurons are core elements of a complex neural network that serves as an important integrative center for regulation of cardiac function. Although mouse models are used frequently in cardiovascular research, very little is known about mouse intrinsic cardiac neurons. Accordingly, we have dissociated neurons from adult mouse heart, maintained these cells in culture, and defined their basic phenotypic properties. Neurons in culture were primarily unipolar, and 89% had prominent neurite outgrowth after 3 days (longest neurite length of 258 ± 20 μm, n = 140). Many neurites formed close appositions with other neurons and nonneuronal cells. Neurite outgrowth was drastically reduced when neurons were kept in culture with a majority of nonneuronal cells eliminated. This finding suggests that nonneuronal cells release molecules that support neurite outgrowth. All neurons in coculture showed immunoreactivity for a full complement of cholinergic markers, but about 21% also stained for tyrosine hydroxylase, as observed previously in sections of intrinsic cardiac ganglia from mice and humans. Whole cell patch-clamp recordings demonstrated that these neurons have voltage-activated sodium current that is blocked by tetrodotoxin and that neurons exhibit phasic or accommodating patterns of action potential firing during a depolarizing current pulse. Several neurons exhibited a fast inward current mediated by nicotinic ACh receptors. Collectively, this work shows that neurons from adult mouse heart can be maintained in culture and exhibit appropriate phenotypic properties. Accordingly, these cultures provide a viable model for evaluating the physiology, pharmacology, and trophic factor sensitivity of adult mouse cardiac parasympathetic neurons.

cholinergic neurons; parasympathetic ganglia; immunohistochemistry; electrophysiology

INTRINSIC CARDIAC NEURONS are core elements of a complex neural network that is localized to the heart and serves as an important integrative center for regulation of cardiac function (3, 22). The interconnected ganglia of this complex have the anatomic designation of cardiac parasympathetic ganglia, and they contain postganglionic parasympathetic neurons, which provide cholinergic innervation of the heart (14, 21). Nevertheless, most intrinsic cardiac neurons have a complex neurochemical phenotype that can include the expression of markers for peptidergic, noradrenergic, and nitrergic neurons in addition to the expected cholinergic markers (16, 22–24, 29, 30, 32). Further indication of heterogeneity among intrinsic cardiac neurons has emerged from in vivo studies of dog and cat hearts. This work has provided evidence that, in addition to cholinergic efferent neurons, the intrinsic cardiac ganglia contain noradrenergic efferent neurons, local circuit neurons, and sensory neurons (3, 7). Other evidence for phenotypic variation within the population of intrinsic cardiac neurons comes from electrophysiological and structural analyses performed in vitro (1, 2, 22). Intrinsic cardiac neurons have been classified as phasic, accommodating/adapting, or tonic depending, respectively, on whether they discharge only one or two action potentials during a constant depolarizing current injection, several action potentials at a decreasing rate, or repetitive action potentials at a constant rate (1, 25). Morphological variation is evident from the identification of unipolar, bipolar, and multipolar intrinsic cardiac neurons in a wide range of species (1, 4, 11).

Thus far, intrinsic neurons of the mouse heart have received relatively little attention in spite of extensive use of mouse models in cardiovascular research. We recently reported that virtually all intrinsic cardiac neurons of adult mice exhibit immunoreactivity for markers of the cholinergic phenotype and for the neuritin (NRTN) receptors (21). Developmental studies have shown that neurotrophic support from NRTN is required for normal cholinergic innervation of the mouse heart (13). Nevertheless, a significant population of cardiac neurons also shows immunoreactivity for noradrenergic markers (15, 32), which suggests that mouse intrinsic cardiac neurons exhibit neurochemical complexity similar to that identified for other species, including humans (29, 32).

To facilitate further evaluation of mouse intrinsic cardiac neurons, we have developed a reliable method for dissociating these cells from adult hearts and maintaining them in primary coculture with other cardiac cells. Using this method, we found that adult mouse intrinsic cardiac neurons thrive in vitro and maintain the cholinergic phenotype but also show evidence of neurochemical, morphological, and electrophysiological heterogeneity. These experiments also provide indirect evidence that nonneuronal cells may provide support for neurite outgrowth by adult intrinsic cardiac neurons.

MATERIALS AND METHODS

Preparation of cover slips. Glass cover slips (12 mm diameter) were passed briefly through a gas flame to enhance coating and then transferred to a 24-well culture plate where they were treated with 500 μg/ml poly-DL-ornithine (Sigma-Aldrich, St. Louis, MO) for 72 h at 4°C followed by 5 μg/ml laminin (In Vitrogen, Carlsbad, CA) for 3 h at 37°C.
Lysine-coated cover slips used in some experiments for preplating were prepared by treating flamed cover slips with 100 μM poly-D-lysine (Sigma-Aldrich) for 3 h at 37°C.

Primary cell culture. Intrinsic cardiac neurons were isolated from the hearts of adult male C57BL/6 mice (7–16 wk old, 22–26 g). Animal protocols were approved by the East Tennessee State University Committee on Animal Care and conformed to the guidelines of the National Institutes of Health published in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85-23, revised 1996). Mice were deeply anesthetized with 5% isoflurane and killed by cervical dislocation. Hearts were removed and placed in ice-cold oxygenated Hanks’ balanced salt solution (HBSS; Sigma-Aldrich) supplemented with 0.1 mM CaCl₂, 10 mM glucose, and 10 mM sodium HEPES, with phenol red serving as an indicator of pH. Mouse intrinsic cardiac ganglia are located primarily near the central portion of the atrium, so this region was isolated by first separating the atria from the ventricles and great vessels and then removing the atrial appendages. The remaining atrial tissue was cut...
into small pieces with iris scissors and dissociated in 2 ml HBSS containing 10 mg/ml collagenase A (Worthington Biochemical, Lakewood, NJ) and 0.25 mg/ml DNase I (Worthington) for 35 min at 37°C. This was followed by addition of 2 mg/ml trypsin (Sigma-Aldrich) and incubation for another 35 min at 37°C. Vials were shaken vigorously every 5 min during both incubations. Cells were washed one time in culture media without CaCl$_2$ and then treated with 1 mg/ml trypsin inhibitor (Sigma-Aldrich) for 15 min at 37°C. After two more washes, cells were suspended in 1 ml culture media, gently triturated using a fire-polished Pasteur pipette coated with 0.05% BSA, and then plated on ornithine- and/or laminin-treated cover slips. The cell suspension obtained from one heart was divided in half and plated on two cover slips for histological experiments. Cells were plated at half that density for electrophysiological experiments to facilitate location of individual neurons. Cells were maintained in minimum essential medium Eagle HEPES modification supplemented with 1 mg/ml BSA, 50 mM CaCl$_2$, 1 mg/ml gentamicin, 2% antibiotic/antimycotic solution (all from Sigma-Aldrich), and 10% FBS (Invitrogen) in a humidified chamber at 37°C with 5% CO$_2$. After 3 h, the cover slips were dipped two times across the cover slip with a BSA-coated glass pipette, then unbound cells, was gently swirled around the well two times, flushed and incuded with 1 ml culture media without CaCl$_2$ and then treated with 1 mg/ml trypsin inhibitor (Sigma-Aldrich) for 15 min at 37°C. After two more washes, cells were suspended in 1 ml culture media, gently triturated using a fire-polished Pasteur pipette coated with 0.05% BSA, and then plated on ornithine- and/or laminin-treated cover slips. The cell suspension obtained from one heart was divided in half and plated on two cover slips for histological experiments. Cells were plated at half that density for electrophysiological experiments to facilitate location of individual neurons. Cells were maintained in minimum essential medium Eagle HEPES modification supplemented with 1 mg/ml BSA, 50 mM CaCl$_2$, 1 mg/ml gentamicin, 2% antibiotic/antimycotic solution (all from Sigma-Aldrich), and 10% FBS (Invitrogen) in a humidified chamber at 37°C with 5% CO$_2$. After 3 h, the cover slips were dipped briefly in HBSS to remove debris and unattached cells and were then placed in fresh media. For cultures maintained >24 h, 10 µM cytosine-β-d-arabinofuranoside (Sigma-Aldrich) was added to reduce the growth of nonneuronal cells. No mitotic inhibitor was added to cultures used for electrophysiology.

For experiments in which the cell suspension was preplated to remove nonneuronal cells, the cell suspension was plated on lysine-coated cover slips for 1 h at 37°C. The media, which contained unbound cells, was gently swirled around the well two times, flushed two times across the cover slip with a BSA-coated glass pipette, then removed and transferred to ornithine- and/or laminin-treated cover slips. Cells were maintained in the same media as those that were not preplated. To inhibit mitosis in remaining nonneuronal cells, 500 µM 5-fluoro-2′-deoxyuridine (dFUR) and 20 µM aphidicolin (both from Sigma-Aldrich) were added 3 h after cells were replated.

**Immunohistochemistry.** After 72 h in culture, cells were fixed for 20 min in PBS containing 4% paraformaldehyde and 0.2% picric acid. Cover slips were rinsed in 0.1 M PBS (pH 7.3), permeabilized in PBS containing 0.4% Triton X-100 and 0.5% BSA, and blocked for 2 h in PBS containing 10% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA), 1% BSA, and 0.4% Triton X-100. Cells were then incubated for 15–18 h in primary antisera generated in different species (Table 1), washed with 0.1 M PBS, and incubated for 2 h with species-specific donkey secondary antibodies conjugated to either AlexaFluor 488 or 555 (Molecular Probes, Eugene, OR) or to Cy3 (Jackson ImmunoResearch). After further rinsing with 0.1 M PBS, cover glasses were inverted onto Citifluor mountant medium (Ted Pella, Redding, CA) on a glass slide and sealed with clear nail polish. Negative control cover slips were processed without primary antibodies.

**Acetylcholinesterase histochemistry.** The histochemical method of Koelle (19) was used to identify acetylcholinesterase (AChE) in some cocultures. Cells were fixed for 10 min at room temperature, and nonspecific cholinesterase was inhibited irreversibly by preincubation in buffer containing 1 µM tetraisopropylpyrophosphoramide (Sigma-Aldrich) for 30 min at 37°C.

**Image acquisition and analysis.** Immunolabeled and stained neurons were viewed and photographed using an Olympus BX41 fluorescence microscope equipped with an Optronics MagnaFire SP charge-coupled device camera. Neuronal cell body area and neurite

![Image](image-url)
length were determined from digital images by using Stereo Investigator/Workstation software (MicroBrightField, Williston, VT). Processes with a length equal to or greater than the diameter of the cell body were considered neurites and were measured using the continuous tracing function. Cell body area was determined by using the Nucleator probe. Neurite and cell body measurements were analyzed using GraphPad Prism 4 (GraphPad Software, San Diego, CA).

Whole cell voltage-clamp technique. Neurons were maintained in culture for at least 24 h before electrophysiological measurements. Cover slips were transferred to an acrylic chamber (Warner, New Haven, CT) on the stage of an Olympus IMT-2 inverted microscope equipped with Hoffman modulation contrast optics. Cells were superfused at room temperature (22–23°C) with a standard external salt solution containing (in mM): 150 NaCl, 6 KCl, 1 MgCl₂, 1.5 CaCl₂, 10 HEPES, and 10 glucose, with pH adjusted to 7.4 with 1 N HCl. Whole cell patch-clamp pipettes were filled with standard internal solution containing (in mM): 140 KCl, 1 CaCl₂, 2 MgCl₂, 11 EGTA, and 5 HEPES, with pH adjusted to 7.2 with 1 N KOH.

Whole cell patch pipettes (4–5 MΩ in the bath solution) were fabricated from borosilicate glass capillaries (1.2 mm OD, 0.68 ID, type EN-1; Garner Glass, Claremont, CA) with a Brown-Flaming horizontal micropipette puller (P-87; Sutter Instruments, San Rafael, CA). Pipette tips were heat polished before use, and a micromanipulator (MO-202; Narishige) fixed to the microscope was used to position pipettes. The whole cell configurations were obtained by standard patch-clamp technique (8). Membrane currents were measured with a patch-clamp amplifier (Axopatch 200B; Molecular Devices, Sunnyvale, CA) with the low-pass Bessel filter (–3 dB) set at 2-kHz. Electrode resistance, cell access resistance, and cell capacitance were compensated electronically before measurements. In some instances where indicated, linear currents were subtracted from current records by the software’s P/4 protocol. Whole cell currents from the patch-clamp amplifier were fed into a Digidata 1322A digital interface connected to a computer (Dell Optiplex GX200 with a Pentium 3 microprocessor) equipped with Clampex 8 software (Molecular Devices). Records were stored either on computer hard drive or on digital audio tape (Dagan, Minneapolis, MN). Ag/AgCl half-cells constituted the electrodes, and an agar bridge (4% wt/vol in external solution) connected the reference electrode to the bath solution. The junction current was zeroed in the cell-attached mode before whole cell access. Electrode and whole cell capacitances along with electrode series resistance were compensated before all measurements. Measurements were made in voltage-clamp and in current-clamp modes. Nicotinic ACh receptor responses were assessed following pressure ejection (40 psi, Picospritzer II; General Valve, Fairfield, NJ) of ACh (1 mM or 100 μM) from a patch pipette (3–5 μm tip diameter). The ejection pipette was positioned ~50 μm from the cell body and, thus, the final ACh concentration was less than in the pipette.

RESULTS

Intrinsic cardiac neurons from adult mice develop neurites in coculture and show varied morphology. When nonneuronal cells were not removed by preplating, neurons comprised only a small percentage of the cells in culture, but they were easily distinguished by their raised soma and prominent nucleus,
most often accompanied by neurite outgrowth after the first night in culture. Morphological features of the cardiac neurons were evaluated in preparations that were immunolabeled for the panneuronal marker protein gene product 9.5 (PGP 9.5). After 3 days in culture, neuronal cell bodies had a surface area of 251 ± 8 (SE) μm² (n = 114), and many produced elaborate processes that frequently terminated on either nonneuronal cells or on other neurons or neuronal processes (Figs. 1 and 2). The longest neurites reached a total length of 258 ± 20 μm (n = 140) after 3 days in culture. Three neuronal types were identified by evaluating cell morphology of 185 neurons from 6 isolations. Unipolar neurons with a single long neurite were most common and comprised 74% of the sample population. Multipolar neurons had several neurites of various lengths and comprised 19% of the cells. The remaining neurons were classified as bipolar since they had a spindle shape with long neurites at opposite ends.

All neurons in coculture show the cholinergic phenotype, but a subpopulation of these cells also exhibit tyrosine hydroxylase immunoreactivity. All neurons retained their cholinergic phenotype in coculture as evidenced by staining for choline acetyltransferase (ChAT), high-affinity choline transporter (CHT), and vesicular ACh transporter (VACHT) in both neuronal cell bodies and their processes (Fig. 1). The cholinergic phenotype was also verified by prominent staining of cell bodies and their processes for AChE (Fig. 1). About 21% of the neurons that were double labeled for PGP 9.5 and ChAT also exhibited strong tyrosine hydroxylase (TH) immunoreactivity (Fig. 3). When primary antisera were eliminated as a control, no immunostaining was observed.

Nonneuronal cells support neurite outgrowth by intrinsic cardiac neurons but are not required for survival. Significant neurite outgrowth occurred in 89% of neurons maintained in coculture (total of 140 neurons from 3 isolations). The presence of nonneuronal cells was reduced substantially by preplating cell suspensions on lysine-coated cover slips for 1 h, plating unattached cells on poly-DL-ornithine/laminin-coated cover slips, and adding dFUR and aphidicolin to the culture medium (Fig. 2, A–C). After 2 days in culture, neurite outgrowth occurred in only 36% of the neurons identified by staining for PGP 9.5. (total of 214 neurons from 3 isolations; Fig. 2). After 3 days in culture, neurons from preplated cell suspensions were significantly smaller (cross-sectional area: 263 ± 4 μm² vs. 303 ± 5 μm², n = 3) and developed shorter neurites (194 ± 9 μm vs. 309 ± 4 μm, n = 3) compared with neurons grown without preplating (Fig. 2, D and E). Preplating also reduced the percentage of neurons that showed TH immunoreactivity (13.7 ± 1.1% vs. 21.1 ± 2.1%, n = 3; Fig. 2F).

Intrinsic cardiac neurons in coculture exhibit voltage-activated fast sodium current and generate action potentials. For electrophysiology, neurons were readily distinguished by their large somas (~20 μm diameter) with distinct nuclei, and projections from the cell bodies often apposed nearby cells (Fig. 4A). Values for passive electrical properties taken from whole cell patch-clamp recordings of cells 1 day in culture are shown in Table 2. The membrane potential of ~43 mV was determined by the zero-current voltage (V-track), and it compared favorably with that reported for rat intracardiac ganglion neurons by sharp glass microelectrodes (25). Whole cell current measurements first were accomplished following membrane voltage clamps to −40 mV from different holding potentials (Fig. 4B). When the holding potential was −80 mV, the voltage step resulted in a large, transient inward current that eclipsed the decrease of steady-state “leakage” current that accompanied the step decrease in transmembrane voltage. This transient inward current inactivated within a few milliseconds in spite of the sustained voltage clamp at −40 mV. When the holding potential was 0 mV, the voltage step to −40 mV resulted in no change in current apart from that expected by the shift in steady-state voltage. These results suggested that the cells display a voltage-dependent inward current consistent with that of the regenerative response found in neurons. To explore this further, we measured membrane currents in response to a series of 20-mV voltage steps from a holding potential of ~80 mV (Fig. 5). A marked increase of inward current resulted at a step depolarization of membrane potential to −40 mV (Fig. 5A). This substantial inward current was transient, inactivating within milliseconds, and its peak magnitude diminished with successive voltage steps toward more positive transmembrane potential (Fig. 5, A and B). Successive depolarizations also resulted in a slower, sustained outward
current whose magnitude increased with the successive steps toward positive transmembrane voltage (Fig. 5, A and B). Again, these findings are consistent with voltage-activated and -inactivated inward current, along with a voltage-activated outward current comprising the regenerative response of mammalian neurons.

To substantiate further that these cells display functional properties of neurons, we evaluated effects of conditions and agents that are known to inhibit inward current and action potentials of neurons. Voltage ramps from \(-100\) to \(100\) mV (400 ms duration) were applied to cells under whole cell-voltage clamp, and corresponding currents were recorded. Outwardly rectifying currents resulted from these voltage ramps. However, a marked transient inward current occurred midway through the ramp at the “critical firing voltage” of \(-40\) mV (results not shown). This inward current was not evoked when the external Na\(^+\) was substituted with an equivalent amount of \(N\)-methyl-D-glucamine (NMDG\(^+\)). Figure 6A shows that a large inward current resulted when membrane voltage was stepped from the holding potential of \(-80\) to \(-40\) mV. Substituting external Na\(^+\) with NMDG\(^+\) markedly reduced this inward current (Fig. 6A). This substitution left only a small, residual inward current that completely disappeared when NMDG\(^+\) was substituted further for all external Ca\(^{2+}\) (Fig. 6A). Restoration of external Na\(^+\) and Ca\(^{2+}\) restored all inward current (Fig. 6A). A summary of the magnitude of the inward currents recorded under these ion substitutions is shown in Table 3. Action potentials were evoked when cells under current clamp were depolarized from \(-80\) to \(-40\) mV (Fig. 6B). Superfusing the cell with tetrodotoxin (TTX; 300 nM)

<table>
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<tr>
<th>(R_{\text{seal}}), GΩ</th>
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<td>3.7±0.6</td>
<td>187±46</td>
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Values are means ± SE. \(R\), resistance; C, capacitance; \(V_{\text{m}}\), transmembrane voltage.

Fig. 5. Voltage-clamp response of a mouse intrinsic cardiac neuron in primary culture. A: overlay of whole cell membrane currents (top) generated by consecutive steps (\(-100\) to \(100\) mV) in membrane potential (bottom). Holding potential = \(-80\) mV. B: peak inward current and plateau outward current (A) plotted as a function of voltage-clamp potential. Holding potential = \(-80\) mV.

Fig. 6. Effects of Na\(^+\) substitution and tetrodotoxin (TTX) on membrane currents and action potentials of mouse intrinsic cardiac neurons in primary culture. A: overlay of consecutive membrane currents plotted as a function of voltage steps from \(-80\) to \(-40\) mV. Black: control record taken with complete external solution; red: Na\(^+\)-free record taken after external Na\(^+\) was substituted with \(N\)-methyl-D-glucamine (NMDG\(^+\)); green: Na\(^+\) and Ca\(^{2+}\)-free taken after external Na\(^+\) and Ca\(^{2+}\) were substituted with NMDG\(^+\); blue: control, restored taken restoration of complete external solution. Linear and capacitative currents were first subtracted from current recordings by the “\(-P/4\)” protocol of the P-Clamp software. B: overlay of consecutive action potentials recorded by current-clamp depolarization of membrane potential from \(-80\) to \(-40\) mV. Control followed by TTX (300 nM) followed by washout of TTX. C: transient inward current and outward currents activated by voltage clamping a cell in successive 5-mV steps from \(-40\) to \(10\) mV (holding potential = \(-80\) mV). Control followed by TTX (300 nM) followed by washout.

Fig. 2. Passive electrical properties of mouse intrinsic cardiac neurons in culture

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blocked voltage activation of an action potential; however, washing out the TTX reversed this blockage (Fig. 6B). TTX inhibited similar voltage activation of transient inward current, but not the activation of outward current, in voltage-clamped cells subjected to successive 5-mV voltage steps from −40 to 10 mV (holding potential: −80 mV; Fig. 6C).

Intrinsic cardiac neurons in coculture can exhibit phasic or accommodating patterns of action potential discharge. We also examined inherent functional properties of intracardiac neurons in primary culture. Of 17 recordings of action potentials generated in current-clamp mode, 13 cells displayed rapidly adapting phasic action potentials during current-clamp depolarization (Fig. 7A). In contrast, four cells displayed accommodating action potentials (Fig. 7B).

CHi evoked a nicotinic receptor-mediated fast inward current. Cells in voltage clamp mode (holding potential = −60 mV) were pulsed with a bolus of ACh to screen for the presence of nicotinic receptors. A rapid transient inward current occurred in about one-half of the neurons challenged with ACh at 1 day in culture (3 of 7 cells with 100 μM ACh, 0.015 ± 0.009 nA/pF; and 3 of 5 cells with 1 mM ACh, 0.014 ± 0.001 nA/pF) and in a majority of neurons at 2–3 days in culture (6 out of 7 cells pulsed with 100 μM ACh, 0.011 ± 0.001 nA/pF). This response to ACh was blocked by treating the cell with hexamethonium (100 μM), whereas the response returned after washing out the hexamethonium (Fig. 8).

**DISCUSSION**

The present study demonstrates that intrinsic cardiac neurons from adult mice can be maintained in primary coculture with other cardiac cells and that these neurons retain basic neurochemical and electrophysiological properties typical of such neurons in vivo. Specifically, these neurons exhibit all aspects of the cholinergic phenotype, develop processes that contact other cells in coculture and, when stimulated, generate action potentials comprising voltage-gated Na⁺ and Ca²⁺ currents. Many neurons also expressed functional nicotinic receptors as evidenced by ACh-evoked fast inward currents that were blocked reversibly by hexamethonium. We have further demonstrated that intrinsic cardiac neurons survive if most nonneuronal cells have been eliminated from the culture but show a marked reduction of neurite outgrowth under this condition. This finding suggests that nonneuronal cells might release molecules that support neurite outgrowth from the intrinsic cardiac neurons.

Cholinergic neurons were identified previously in cocultures of embryonic mouse heart but only in preparations from hearts collected at 9 days in utero (20). Viable neurons were not detected in cocultures from later embryonic ages or from 1-day postnatal hearts. Although it was suggested that older neurons might be unable to survive the culture procedure, our results show that this is not the case. The use of a different method for tissue dissociation is probably a major factor in obtaining viable neurons from adult mouse hearts. Furthermore, our cultures were prepared from a small region of atrium where...
most intrinsic cardiac ganglia are located, whereas the entire heart was used in the earlier study. This factor should increase the yield of neurons in coculture. Other investigators have shown that intrinsic cardiac neurons can be dissociated and cultured from adult dog hearts (28), neonatal and adult guinea pig hearts (9, 17, 18) and from neonatal and juvenile rat hearts (11, 33).

Functional criteria were used to establish the cholinergic phenotype of intrinsic cardiac neurons obtained from embryonic hearts (20). These preparations contained clusters of beating myocytes that had visible connections with clusters of neurons. The neurons had a tonic inhibitory effect on the myocytes, since beating frequency increased after careful removal of the neuron clusters or addition of 100 nM TTX to the medium. The beating rate also increased after muscarinic receptor blockade with atropine, providing definitive evidence for the cholinergic phenotype of the neurons. Although a majority of the cell population in our cocultures was nonneuronal (e.g., fibroblasts and Schwann cells), beating myocytes were rarely seen and never observed after the first day in culture. The absence of beating myocytes in our preparations and the low abundance of such cells in cocultures of neonatal guinea pig intrinsic cardiac neurons (9) are most likely due to the Ca\textsuperscript{2+} paradox phenomenon in which myocytes are damaged as a result of exposure to Ca\textsuperscript{2+}-free medium followed by the return to a Ca\textsuperscript{2+}-containing medium (26). Accordingly, we used immunohistochemical criteria to establish the cholinergic phenotype of adult intrinsic cardiac neurons in coculture. These experiments showed that cultured neurons have the full complement of proteins required for uptake of choline (CHT), synthesis of ACh (ChAT), and the transport of ACh into storage vesicles (VAChT). They also exhibited staining for AChE, which is characteristic of cholinergic neurons and essential for terminating cholinergic neurotransmission.

Recent studies have shown that a subpopulation comprising ~30% of adult mouse intrinsic cardiac neurons expresses the noradrenergic marker TH in situ (15, 32), and we found that this trait was retained by a similar percentage of neurons in coculture. The functional significance of this characteristic is unknown, but many intrinsic cardiac neurons of the human heart exhibit a full complement of noradrenergic proteins in addition to the cholinergic markers that are expected for parasympathetic neurons (32). It has been suggested that this feature might allow the neurons to switch between cholinergic and noradrenergic functions depending on differential trophic factor exposure (32). The precedent for such a scheme comes from work with cocultures of neonatal sympathetic neurons and beating ventricular myocytes (34). This work demonstrated that brain-derived neurotrophic factor can cause a rapid switch of neurotransmitter phenotype such that noradrenergic control of myocyte function was replaced by cholinergic control.

Neurotrophic factors are essential for the development of the parasympathetic nervous system (12, 13, 27) but may not be required for survival of postganglionic cholinergic neurons in adults. This view is supported by our findings and the work of other investigators who maintained intrinsic cardiac neurons in culture medium supplemented with serum but without addition of neurotrophic factors (5, 9–11, 18). Nonetheless, adult parasympathetic neurons can still respond to neurotrophic factors. NRTN, a member of the glial cell line-derived (GDNF) family of neurotrophic factors, stimulates neurite outgrowth, increases neuronal cross-sectional area, and affects neurochemical phenotype in cultures of adult rat sacral parasympathetic neurons (31). Recent work has also shown that NRTN and GDNF can modulate neuropeptide expression by adult guinea pig neurons in explant culture of intrinsic cardiac ganglia (6). Nonneuronal cells might be a source of neurotrophic factor in our experiments, since removal of these cells caused a drastic reduction of neurite outgrowth, a decrease in cell body area, and a reduction in the percentage of neurons that exhibit TH immunoreactivity.

The adult intrinsic cardiac neurons in our cocultures also had electrical properties reflecting the neuronal phenotype (1, 25). Specifically, these cells exhibited a voltage-dependent fast inward current that was mediated by Na\textsuperscript{+} and blocked by TTX, a slower voltage-dependent outward current, and generation of action potentials. Mouse intrinsic cardiac neurons had either phasic or accommodating firing patterns during depolarizing current injections, which is typical of most intrinsic cardiac neurons of other species (1, 25). Neurons with a tonic firing pattern were not detected, but this may be a limitation of the sample size. Last, ACh evoked a rapid inward current in most intrinsic cardiac neurons at ~2–3 days in coculture, and this response was mediated by nicotinic receptors. Fast nicotinic currents are a defining characteristic of intrinsic cardiac neurons and other postganglionic neurons of the autonomic nervous system. The lack of response to pressure pulses of 100 μM and 1 mM ACh in about one-half of the neurons at 1 day in coculture might be because of damage of their nicotinic receptors during the enzymatic dissociation process. The higher response rate at ~2–3 days in culture supports this conclusion since a longer duration of culture would provide time for synthesis of new receptor protein.

In conclusion, parasympathetic cardiac neurons were dissociated from adult mouse hearts and maintained in coculture where they retained many neurochemical and electrophysiological characteristics that are typical of intrinsic cardiac neurons. This preparation will enable detailed analysis of the pharmacology and electrophysiology of mouse intrinsic cardiac neurons. We anticipate that enriched cultures of intrinsic cardiac neurons will be a suitable model for evaluating the influence of neurotrophic factors on adult mouse cardiac parasympathetic neurons.

GRANTS

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