

Cultivation in Rotating Bioreactors Promotes Maintenance of Cardiac Myocyte Electrophysiology and Molecular Properties

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ABSTRACT

We tested the hypothesis that cardiomyocytes maintained their phenotype better if cultured as three-dimensional tissue constructs than if cultured as confluent monolayers. Neonatal rat cardiomyocytes were cultured on biomaterial scaffolds in rotating bioreactors for 1 week, and resulting tissue constructs were compared with confluent monolayers and slices of native ventricular tissue with respect to proteins involved in cell metabolism (creatine kinase isoform MM), contractile function (sarcomeric myosin heavy chain), and intercellular communication (connexin 43), as well as action potential characteristics (e.g., membrane resting potential, maximum depolarization slope, and action potential duration), and macroscopic electrophysiological properties (maximum capture rate). The molecular and electrophysiological properties of cardiomyocytes cultured in tissue constructs, although inferior to those of native neonatal ventricles, were superior to those of the same cells cultured as monolayers. Construct levels of creatine kinase, myosin heavy chain, and connexin 43 were 40–60% as high as ventricle levels, whereas monolayer levels of the same proteins were only 11–20% as high. Construct action potential durations were 1.8-fold higher than those in ventricles, whereas monolayer action potential durations were 2.4-fold higher. Pharmacological studies using 4-aminopyridine showed that prolonged action potential duration and reduced maximum capture rate in tissue constructs as compared with native ventricles could be explained by decreased transient outward potassium current.

INTRODUCTION

CARDIOMYOCYTES CULTURED IN MONOLAYERS exhibit properties different from native heart tissue,^{1,2} because of the effects of cell isolation and *in vitro* cultivation, and because of structural differences between two- and three-dimensional (2-D and 3-D) environments.³ Tissue constructs obtained by bioreactor cultivation of cardiomyocytes on 3-D scaffolds could potentially be used

in vitro, as a model system for electrophysiological and pharmacological studies, or *in vivo*, as grafts to repair damaged cardiac muscle. Researchers in several groups have generated cardiac tissue constructs starting from cardiomyocytes, biomaterial scaffolds, and in some cases bioreactors.^{4–12} Dissociated cardiomyocytes^{13–15} and tissue constructs^{16–20} were implanted *in vivo* in an attempt to improve cardiac function in myocardial injury models, but whether the repair tissue consisted of a continu-

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ous syncytium with normal action potentials and impulse propagation has not yet been evaluated.

In previous *in vitro* studies, the structure and function of cardiac tissue constructs depended on the cell source and preparation,^{5,6} initial cell-seeding density,⁶ the scaffold and culture medium,¹⁰ the bioreactor,^{6,7,10} and applied physical forces.^{9,12} Ideally, a 3-D scaffold for cardiac tissue engineering should (1) provide a structural template that can permit seeded cardiac myocytes to undergo cardiogenesis *in vitro*, (2) promote integration of engineered constructs with native cardiac tissue after *in vivo* implantation, and (3) biodegrade, in order to minimize immunogenicity. After scaffold degradation, construct structural integrity will depend on cell-to-cell connectivity, a small amount of connective tissue, and any host-derived vascularization that may develop after *in vivo* implantation. A variety of scaffolds are currently under investigation for cardiac tissue-engineering applications, including polyglycolic acid (PGA) nonwoven mesh with or without laminin coating,^{5,6,10} collagen hydrogel with or without Matrigel,^{8,12,19} collagen fibers,⁴ and collagen sponge with or without Matrigel.^{18,21}

Constructs generated in our laboratory had an approximately 100- μ m-thick outer layer that supported macroscopically continuous isotropic impulse conduction at a velocity of 20 cm/s (comparable to neonatal ventricles), captured at maximum rates of 300 beats/min (bpm, 65% as high as neonatal ventricles), and contained a spatially uniform distribution of connexin 43.¹⁰

The present study tested the hypothesis that cardiac myocytes maintain their electrophysiological and molecular properties better if cultured on 3-D scaffolds in bioreactors than if cultured as monolayers. Neonatal rat cardiomyocytes were cultured on PGA scaffolds in rotating bioreactors for 1 week, and the resulting tissue constructs were compared with confluent monolayers and with slices of native neonatal rat ventricular tissue with respect to (1) proteins involved in cell metabolism (creatine kinase isoform-MM, CK-MM), contractile function (sarcomeric myosin heavy chain, MHC), and intercellular communication (connexin 43, Cx-43), and (2) action potential characteristics (e.g., membrane resting potential or MRP; maximum depolarization slope or MDS; action potential duration or APD) and macroscopic electrophysiological properties (maximum capture rate or MCR). Pharmacological studies were done in an attempt to explain some of the observed electrophysiological differences.

MATERIALS AND METHODS

Materials

Materials were from Sigma (St. Louis, MO) or GIBCO (Grand Island, NY), unless otherwise specified.

Methods

Cell and tissue culture

Cell isolation. Cardiomyocytes were isolated from the lower portions of ventricles of neonatal (2-day-old) Sprague-Dawley rats (Taconic, Germantown, NY) by incubation in trypsin followed by serial digestion with collagenase type II (Worthington Biochemical, Lakewood, NJ).⁵ Cells were preplated in two 45-min steps and suspended in Dulbecco's modified Eagle's medium (DMEM) containing glucose (4.5 g/L), 10% fetal bovine serum (FBS; HyClone, Logan, UT), 10 mM HEPES, 4 mM L-glutamine, and penicillin (100 U/mL).

Monolayers. Glass coverslips (12 \times 40 mm; Fisher Scientific International, Hampton, NH) were surface hydrolyzed by incubation in 1 M NaOH for 1 min, coated by incubation in laminin (6.5 μ g/mL) overnight at 37°C, and seeded with 1.5×10^5 cells/cm² (Fig. 1). On culture day 2, the serum was switched from 10% FBS to 2% horse serum, and the medium was replaced every other day for 1 week. A total of 36 monolayers from 6 independent studies were analyzed.

Tissue constructs. Engineered tissue constructs were prepared as previously described (Fig. 1).¹⁰ In brief, discs (5 mm in diameter \times 2 mm thick) of polyglycolic acid (PGA) mesh (Albany International Research, Mansfield, MA) were surface hydrolyzed, laminin coated, ethylene oxide sterilized, and placed in 96-well plates. Concentrated cells were added (8 million cells in 60 μ L of medium per disc), and plates were mixed at 25 rpm in a humidified 37°C/5% CO₂ incubator. After 1 h, six constructs and the remaining cells in the wells were transferred into bioreactors (100-mL high aspect ratio vessel (HARV); Synthecon, Houston, TX) that were rotated at 12 rpm in order to maintain the constructs freely suspended. The serum in the medium was switched on culture day 2 from 10% FBS to 2% horse serum, and medium was replaced by 50% on culture days 4 and 6. A total of 36 constructs from 6 separate studies were analyzed.

Neonatal ventricles. Midmyocardial slices (approximately 5 \times 4 mm², 0.45 mm thick) obtained from the ventricular walls of 2-day neonatal rats, using a Vibratome (Ted Pella, Redding, CA), were used for intracellular electrophysiological studies. Full-thickness slices (approximately 6 \times 4.5 mm², 1.5 to 2.5 mm thick), obtained by bisecting the ventricle parallel to the base apex line, were used for extracellular electrophysiological studies.

Structural, biochemical, and molecular assessments

Structural assays. Monolayers were fixed with Histochoice (Amresco, Solon, OH) for 30 min, immunostained

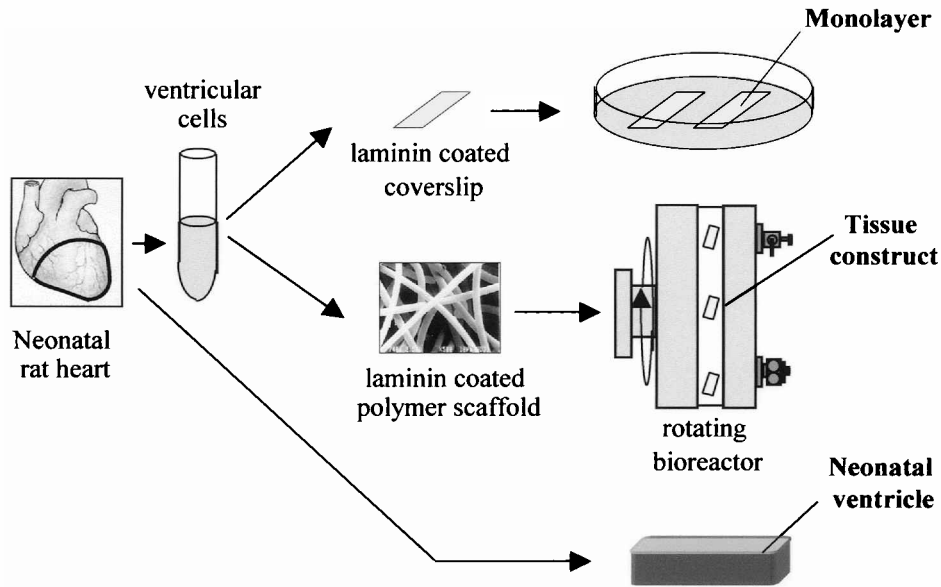


FIG. 1. Experimental design. Neonatal rat cardiac cells were cultured for 1 week as monolayers (on laminin-coated coverslips) or as tissue constructs (on laminin-coated PGA mesh in bioreactors). Slices of neonatal rat ventricles served as controls.

with mouse anti-Cx-43 monoclonal antibody (Chemicon International, Temecula, CA), and assessed by confocal fluorescence microscopy.¹⁰ In the absence of Cx-43 antibody, no nonspecific fluorescence was observed. Tissue constructs and neonatal ventricles were fixed with formalin, embedded in paraffin, sectioned to 5 μm thick, and stained with hematoxylin and eosin (H&E) and with anti-Cx-43 antibody.

Biochemical assays. DNA and protein assays were carried out as previously described.¹⁰ In brief, cells were dissociated from monolayers by trypsin; tissue constructs and neonatal ventricles were homogenized. DNA content was assessed with Hoechst dye 33258 (Polysciences, Warrington, PA) and a spectrofluorimeter (Photon Technology International, Lawrenceville, NJ), and protein content was assessed with a Bio-Rad (Hercules, CA) DC assay kit and a microplate spectrophotometer (MR5000; Dynatech, Chantilly, VA).

Molecular assay. Western blot was used to quantify relative amounts of Cx-43, MHC, and CK-MM as previously described.¹⁰ In brief, monolayers were incubated with PBS containing protease inhibitors and cells were detached with a cell scraper; tissue constructs and neonatal ventricles were homogenized. Proteins were eluted, electroblotted, blocked for nonspecific antibodies, incubated with primary antibody, and conjugated to horseradish peroxidase. Primary antibodies were rabbit anti-Cx-43 (Zymed, South San Francisco, CA), goat anti-CK-MM (Biosdesign International, Saco, ME), and mouse anti-MHC (Developmental Studies Hybridoma

Bank). Immunocomplexes were developed and detected with photographic film. Band intensity was measured by laser scanning densitometry (Molecular Dynamics; Amersham Biosciences, Piscataway, NJ). To compare protein band intensities between two gels, data were normalized to a common sample loaded in triplicate on each gel.

Electrophysiological assessments

Intracellular recordings. Whole cell voltage clamping of cardiomyocytes harvested enzymatically from 7-day constructs could not be carried out because of low membrane resistance. Ionic currents were therefore assessed indirectly by recording the propagated action potentials of impaled cells within intact samples (cell monolayers, tissue constructs, and slices of neonatal ventricles) as follows.

The sample was positioned in a perfused chamber (Harvard Apparatus, Holliston, MA) and superfused with oxygenated Tyrode's solution (140 mM NaCl, 5.4 mM KCl, 0.33 mM NaH_2PO_4 , 1.8 mM CaCl_2 , 1 mM MgCl_2 , 5 mM HEPES, 5.5 mM D-glucose; pH adjusted to 7.4) at room temperature. Extracellular stimulation was applied with a pair of tungsten electrodes (50- μm tip diameter; Microprobe, Carlsbad, CA) positioned 200 μm apart and connected to a programmable cardiac stimulator (SEC-3102; Nihon Kohden, Foothill Ranch, CA). A sharp glass microelectrode was drawn on a horizontal pipette puller (P-97; Sutter instrument, Novato, CA) to achieve an impedance of 50 M Ω when filled with 2 M KCl. The microelectrode was advanced either into a cell

in an intact monolayer, or into a cell within an intact tissue construct or ventricular slice at a depth of 10 to 100 μm . Recordings were made at least 2.5 mm away from the stimulus site to avoid stimulus-induced artifacts. Excitation-contraction decoupling agents were not used; intracellular recordings that exhibited motion artifacts (approximately 10–20% of all recordings) were not analyzed.

Tissue constructs and neonatal ventricles were superfused for 5 min and stimulated at 60 bpm (pulses of 0.4–4.0 V, 2 ms), and propagated action potentials were recorded for 5 min from $n \geq 6$ different cells randomly selected throughout each sample. In monolayers, action potentials were recorded from cells that were spontaneously contracting at rates comparable to those in stimulated 3-D tissues in order to minimize rate-dependent effects on action potential shape.²² For pharmacological studies, baseline recordings were obtained from $n \geq 6$

cells in a small tissue area ($<1 \text{ mm}^2$). Subsequently, samples were superfused for 30 min with a test drug and a second set of recordings were made from $n \geq 5$ cells in the same tissue area. The drugs studied were palmitoleic acid (10 μM in Tyrode's solution)²³ and 4-aminopyridine (4-AP, 4 or 12 mM in Tyrode's solution).^{24,25}

Recorded signals were amplified with an Axoclamp 2B amplifier (Axon Instruments, Union City, CA) and low-pass filtered with an eighth-order Butterworth filter with a cutoff frequency of 5 kHz. Filtered signals were displayed on an oscilloscope, digitized at a sampling rate of 10–20 kHz using a 32-bit A/D board (DAP 3200a; Microstar Laboratories, Bellevue, WA), and real-time displayed and stored using LabView (National Instruments, Austin, TX), and analyzed using MATLAB (MathWorks, Natick, MA). Recorded action potentials were characterized by measuring MRP, MDS, and APD at 10, 20, 50, and 90% of repolarization, and action potential amplitude

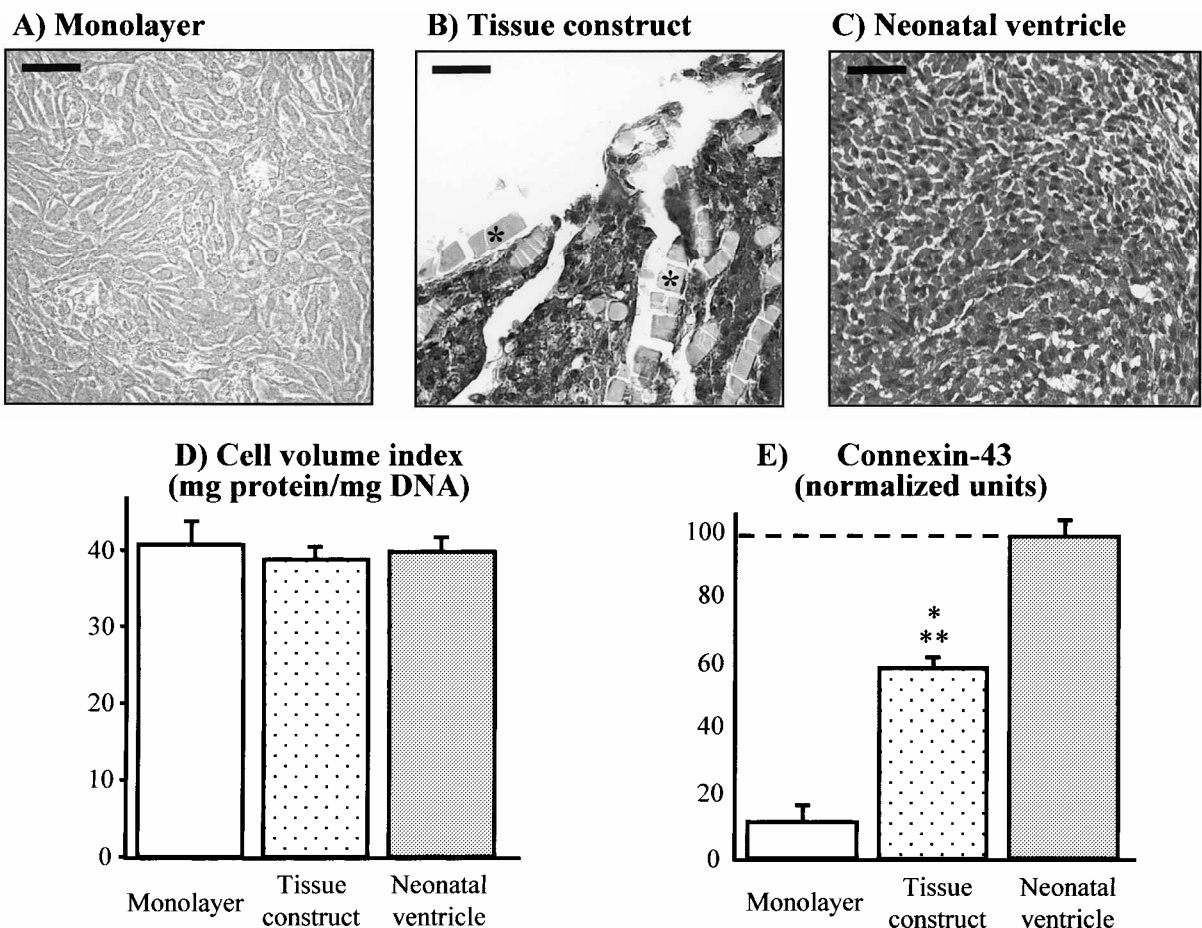


FIG. 2. Cell morphology, volume, and intercellular coupling. Appearance of a representative (A) monolayer, (B) tissue construct, and (C) neonatal ventricle assessed by (A) phase-contrast microscopy and (B and C) H&E-stained histological sections. *Polymer fiber. Scale bars: (A) 50 μm ; (B and C) 20 μm . (D) Index of cell volume (mg total protein/mg DNA); (E) connexin-43 level, assessed by scanning of Western blots (normalized per unit of total protein and expressed as a fraction of that in neonatal ventricles). Data represent the average \pm SEM of nine independent measurements. *Significantly different from neonatal ventricles, $p < 0.0001$; **significantly different from monolayers, $p < 0.0001$.

(APA). Results for each parameter obtained from different cells in a single sample were averaged. For pharmacological studies, responses of baseline cells in a small tissue area were averaged compared with responses of drug-treated cells from the same area. This approach was validated by the finding that different cells within a small tissue area exhibited action potentials with virtually identical shapes.

Extracellular recordings. Tissue constructs and neonatal ventricles were positioned in a custom-built test chamber⁵ and superfused with oxygenated Tyrode's solution at 37°C. Extracellular stimulation was applied at a rate of 60 bpm, using a pair of tungsten electrodes and a programmable cardiac stimulator, and extracellular recordings were made with a linear array of eight electrodes positioned 1.5 to 5 mm from the stimulation site.⁵ The stimulation rate was increased progressively to measure the maximum stimulation frequency at which the sample could be captured for 5 min (MCR).⁵ Baseline MCRs were compared with the MCR measured 30 min after treatment with two different concentrations of 4-AP.

Statistics

Data were expressed as means \pm SEM and analyzed using either a paired *t* test or one-way analysis of variance (ANOVA) followed by Fisher's protected least significance difference posthoc test. Differences were considered statistically significant when $p < 0.05$. Analysis was performed with SuperANOVA III for the Macintosh.

RESULTS

Cell morphology, coupling, and expression of differentiation markers

Cell size and a cell volume index (i.e., mg protein/mg DNA) were comparable for 7-day monolayers, 7-day tis-

sue constructs, and native neonatal ventricles (Fig. 2). Our structural and functional analyses of tissue constructs focused on their peripheral zones, that is, the approximately 0.1-mm-thick surface zone in which the cells appeared interconnected, randomly distributed, and exhibited a variety of morphologies, from elongated and spread on or between the polymer fibers, to round and unattached (Fig. 2B, and Refs. 5 and 10). As previously documented, in this peripheral zone the tissue was electrophysiologically functional⁵ and cardiac myocytes formed new cell-to-cell junctions that were punctate and uniformly distributed¹⁰ and exhibited ultrastructural features characteristic of cardiac muscle.^{5,10} As in our previous studies, before those in which constructs were cultured in perfused cartridges,^{7,21} tissue constructs had central regions that were relatively acellular (data not shown) and peripheral zones that had lower cellularity than neonatal ventricles (Fig. 2B versus Fig. 2C).

Tissue constructs expressed Cx-43, CK-MM, and MHC, at levels between those of monolayers and neonatal ventricles (Fig. 2E and Fig. 3A and B). Constructs expressed Cx-43 at levels 60% as high as ventricles, whereas monolayers expressed the same marker at levels only 11% as high (Fig. 2E). Constructs expressed CK-MM and MHC at levels 45–50% as high as those of ventricles, whereas monolayers expressed the same markers at levels only 13–20% as high (Fig. 3A and B).

Electrophysiological function

Tissue constructs exhibited spontaneous contractile activity at the early stages (e.g., days 2–4) of culture. However, spontaneous contractions ceased by day 7, as previously reported.^{5,10} In contrast, monolayers contracted synchronously at rates ranging from 30 to 160 bpm, starting on day 1–2 and continuing through day 7.

Tissue constructs exhibited isotropic electrophysiological properties, that is, on electrical stimulation with a

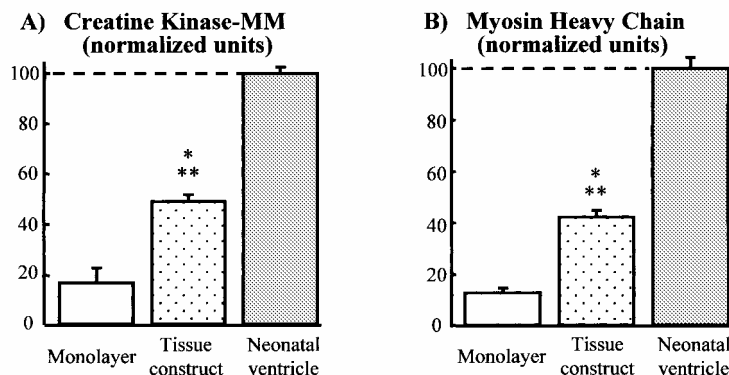


FIG. 3. Molecular markers of differentiation: Levels of (A) creatine kinase-MM and (B) sarcomeric myosin heavy chain, as assessed by scanning of Western blots (normalized per unit of total protein and expressed as a fraction of that in neonatal ventricles). Data represent the average \pm SEM of nine independent measurements. *Significantly different from neonatal ventricles, $p < 0.0001$; ** significantly different from monolayers, $p < 0.0001$.

bipolar point electrode, tissue in the zone at the construct periphery supported isotropic electrical propagation at a velocity comparable to that measured for neonatal ventricles.^{5,10} Isotropic electrophysiological properties were consistent with the observed tissue architecture, that is, the interconnected, randomly distributed cells in the peripheral zone of constructs did not exhibit cellular alignment on a macroscopic scale, a finding that may be attributed to the randomly orientated nonwoven fibers in the PGA scaffold.

In tissue constructs and neonatal ventricles, the mechanical contractions that followed propagated electrical activity were visible to the naked eye and recorded action potentials were characteristic of electrically quiescent nonpacemaker (NP) myocytes, that is, a steady resting potential and a high slope of depolarization of >75 V/s (Fig. 4B and C). In contrast, in monolayers approximately 10–20% of the examined cells generated action potentials characteristic of electrical pacemaker (P) cells, that is, a resting potential that steadily depolarized toward a threshold of -55 to -65 mV and a low slope of depolarization of <35 V/s (Fig. 4A). Pacemaker cells established the spontaneous contraction rate of the monolayer, which was approximately constant for a given sample but varied between samples over a wide range (30–160 bpm). Addition of 0.5 mM CdCl₂, a calcium channel blocker, to the monolayer terminated whereas washout of this drug reinitiated spontaneous contractility

(data not shown). Action potentials in the remaining 80–90% of the examined cells in monolayers were characteristic of NP cells (Fig. 4A).

The MRP values of NP cells were comparable in all three experimental groups and averaged -72 ± 0.4 mV (Fig. 4D), a value comparable to that previously reported for neonatal rat cardiomyocytes that were freshly isolated²⁶ or cultured in monolayers.^{2,26,27} The MDS of NP cells was similar for monolayers and tissue constructs, and 85% as high as that in neonatal ventricles (Fig. 4E). The APA was similar for monolayers and tissue constructs, comparable to the value previously reported for monolayers of neonatal rat cardiomyocytes,^{26,27} and slightly but significantly higher than that measured for neonatal ventricles (Fig. 4F). Tissue construct APDs were in between those of monolayers and native ventricles at all phases of repolarization (Fig. 4G). In particular, tissue constructs exhibited APD₂₀, APD₅₀, and APD₉₀ values that were, respectively, 2.4-, 1.9-, and 1.8-fold longer than those of neonatal ventricles, whereas in monolayers APD₂₀, APD₅₀, and APD₉₀ values were, respectively, 3.3-, 2.5-, and 2.4-fold longer than those of ventricles.

An intercellular decoupling agent, palmitoleic acid (10 μ M, 20-min exposure) slowed impulse propagation but did not significantly change the APD (data not shown). In contrast, 4-aminopyridine (4 mM, 30-min exposure), a blocker of the transient outward potassium current (I_{to}), increased the APD₁₀, APD₅₀, and APD₉₀ in tissue con-

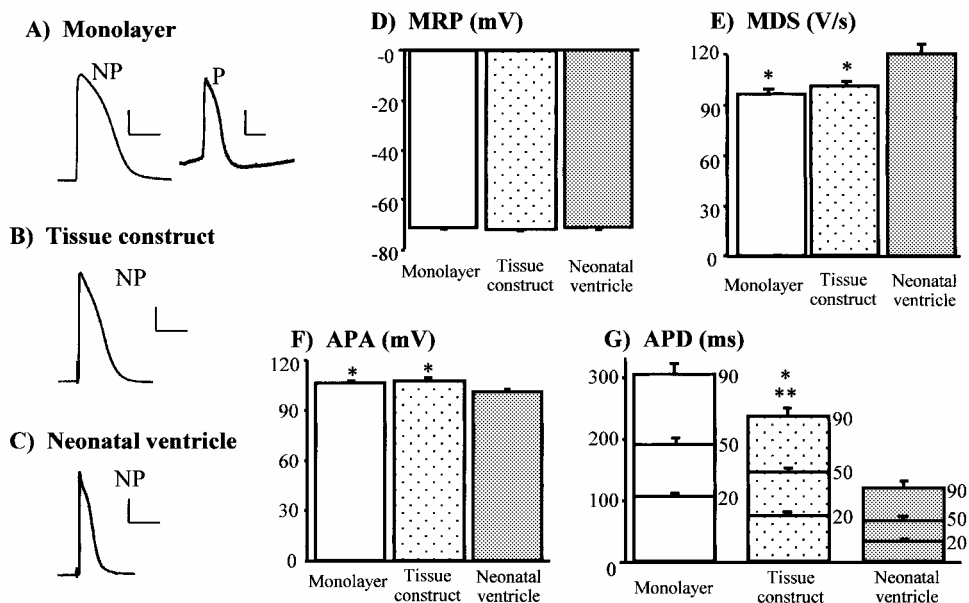


FIG. 4. Electrophysiological parameters. Representative action potentials recorded from (A) monolayers, (B) tissue constructs, and (C) neonatal ventricles. Nonpacemaker and pacemaker cells are, respectively, designated NP and P; horizontal and vertical scale bars are, respectively, 200 ms and 25 mV. (D) Membrane resting potential (MRP, mV), (E) maximum depolarization slope (MDS, V/s), (F) action potential amplitude (APA, mV), and (G) action potential duration (APD, ms) at 20, 50, and 90% of repolarization shown for NP cells. Data represent the average \pm SEM of 18 independent measurements. *Significantly different from neonatal ventricles, $p < 0.001$; ** significantly different from monolayers, $p < 0.001$.

structs and neonatal ventricles, but not in monolayers (Fig. 5). Longer exposure times induced no additional effects, implying 30 min was sufficient for drug equilibration within the sample. In tissue constructs and neonatal ventricles, effects of 4-AP were most pronounced in the early stage of repolarization (i.e., APD₁₀; Fig. 5). In neonatal ventricles, 4 mM 4-AP resulted in an apparent increase in the intensity of contractions and an associated increase in motion artifacts that decreased the percentage of successful recordings and might be explained by an increased calcium influx.²⁸

Neonatal ventricles exhibited dose-dependent increases in APD₂₀, APD₅₀, and APD₉₀ and a dose-dependent decrease in MCR in the presence of 4-AP (Fig. 6A). At a concentration of 12 mM, 4-AP increased the APD₂₀, APD₅₀, and APD₉₀ of neonatal ventricles to match the corresponding APD measured for baseline (i.e., drug-free) tissue constructs (Fig. 6A). Likewise, 12 mM 4-AP decreased the MCR of neonatal ventricles to match the MCR measured for baseline tissue constructs (305 bpm) (Fig. 6B). Values of the other electrophysiological parameters studied were not significantly affected by 4-AP (data not shown).

DISCUSSION

The major finding of the present study was that molecular and electrophysiological properties of neonatal rat cardiac myocytes cultured on 3-D scaffolds in rotating bioreactors, although inferior to those of native neonatal ventricles, were superior to those of the same cells cul-

tured as monolayers. In particular, construct levels of CK-MM, MHC, and Cx-43 were 40–60% as high as those of ventricles, whereas monolayer levels were only 11–20% as high, and APDs were less prolonged in tissue constructs than in monolayers. Pharmacological studies showed that prolonged APD and reduced MCR in tissue constructs as compared with native ventricles could be explained by a decrease in I_{to} .

A wide variety of cell types exhibit enhanced maintenance of their differentiated phenotype if cultured in 3-D systems instead of monolayers, which has been attributed to associated differences in cell shape and/or increases in intercellular communication.^{3,29,30} Consistent with the above observations, the levels of proteins involved in cell metabolism, contractile activity, and intercellular communication in tissue constructs were in between those of native ventricles and monolayers (Figs. 3 and 2E). It was previously shown that neonatal rat ventricular myocytes in confluent monolayers couple on average with six cells, whereas the same cells in native ventricles couple on average with nine cells.³¹ Lower numbers of cell-to-cell contacts may therefore explain why monolayers had lower levels of Cx-43 than did tissue constructs (Fig. 2E), and, in conjunction with lower cell densities (Fig. 2B and C), may explain why tissue constructs had lower levels of Cx-43 than did native ventricles (Fig. 2E). The finding that neonatal rat heart cells did not increase in volume (based on total protein per unit DNA) over 7 days of culture in either monolayers or constructs (Fig. 2D) may be due to the use of medium with a low serum concentration (2%). In contrast, an increase in cell size has been reported for neonatal rat heart

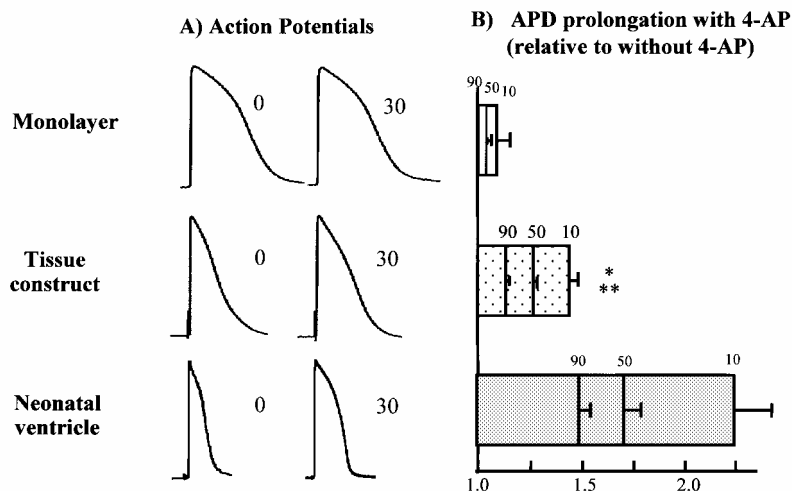


FIG. 5. Effect of 4-aminopyridine (4-AP) on action potential duration (APD). (A) Action potentials recorded before and 30 min after application of 4 mM 4-AP, denoted, respectively, by 0 and 30. (B) Graphic representation of the APD 30 min after application of 4 mM 4-AP normalized relative to the APD before drug application at 10, 50, and 90% of repolarization. Data represent the mean \pm SEM of nine independent measurements. *Significantly different from neonatal ventricles, $p < 0.001$; **significantly different from monolayers, $p < 0.01$.

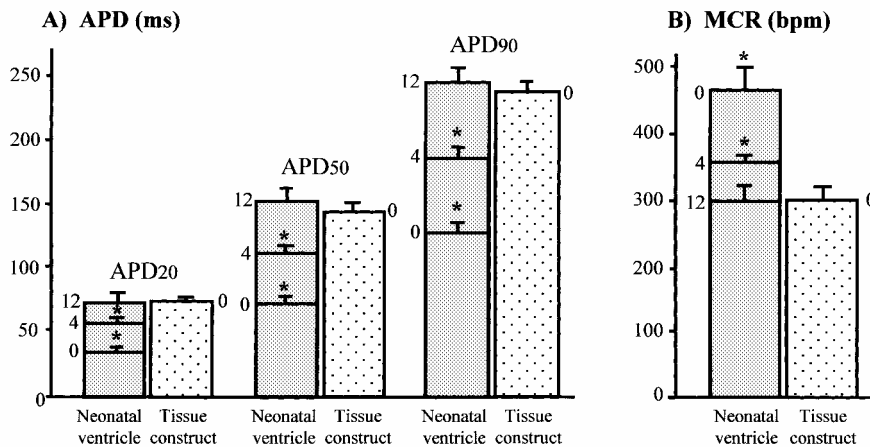


FIG. 6. Effect of 4-aminopyridine (4-AP) concentration on action potential duration (APD) and maximum capture rate (MCR) in neonatal ventricles. **(A)** APD at 20, 50, and 90% of repolarization and **(B)** MCR for samples exposed for 30 min to 4-AP at concentrations of 0, 4, and 12 mM (0, 4, and 12, respectively). Data represent the mean \pm SEM of nine independent measurements. *Significantly different from tissue construct, $p < 0.01$.

cells cultured with a high serum concentration (10%) in monolayers³² but not in 3-D tissue constructs.^{5,6}

In contrast to tissue constructs and neonatal ventricles, 7-day monolayers exhibited spontaneous pacemaker activity, that is, slow calcium-dependent action potentials characteristic of embryonic and dedifferentiated cardiomyocytes.² Observed pacemaker cells were not likely to be of nodal origin, because cells were harvested from the lower portion of the donor ventricles. The apparent absence of pacemaker cells in tissue constructs and neonatal ventricles could be due to better maintenance of the resting membrane potential in 3-D than 2-D cultures, or to the fact that the pacemaker cells in 3-D cultures experienced a larger electrotonic load due to better coupling (Fig. 2E), and thus could not generate sufficient source current to drive neighboring cells.³³ This explanation is supported by the occasional presence of spontaneous contractile activity in constructs during the early stages of culture.

The MRP of nonpacemaker cells in monolayers, tissue constructs, and neonatal ventricles were comparable (Fig. 4D), implying a similar density of inward rectifier K^+ current (I_{K1})^{2,34} and a relatively high level of electrical differentiation.² In addition, the MDS values of cells in monolayers and constructs were 90 to 100 V/s (Fig. 4E), that is, at the high end of those previously reported.^{2,27} The MDS of a propagated action potential is known to depend on many factors including the fast Na^+ current,³⁵ and tissue architectural features, such as resistances of the intracellular and extracellular spaces, cell size, and gap junctional distribution.³⁶ Constructs had significantly lower MDS values than did ventricles (Fig. 4E) but similar conduction velocities,¹⁰ suggesting that Na^+ currents probably did not differ significantly,^{35,36}

and that observed differences in MDS were due mainly to differences in tissue architecture.

Tissue constructs exhibited APDs in between those of monolayers and native ventricles (Fig. 4G). APD prolongation and attenuation of the fast repolarization phase observed in constructs compared with ventricles were apparently not due to a difference in electrical coupling, because APD prolongation was not induced by the decoupling agent palmitoleic acid, but rather to a difference in I_{to} , as demonstrated with 4-AP (Figs. 5 and 6). I_{to} , a major repolarizing current in neonatal rat ventricular myocytes,²⁴ activates with cell depolarization, determines both early and later stages of repolarization, and is regulated by $Kv4.2$ and $Kv4.3$ gene expression.^{37,38} The main component of I_{to} is rapidly inactivating, 4-AP-sensitive, and Ca^{2+} -independent current.³⁸ A reduction of I_{to} is known to prolong APD in different pathological states, such as chronic myocardial infarction³⁹ and different forms of hypertrophy and heart failure.²⁵

As compared with neonatal ventricles, tissue constructs exhibited attenuated, 4-AP sensitive I_{to} , whereas monolayers exhibited no significant response to 4-AP (Fig. 5); the latter finding is consistent with previous studies of neonatal rat myocytes²⁶ and adult cat myocytes.⁴⁰ In tissue constructs and ventricles, the most pronounced effect of 4-AP on APD was seen at the earliest stages of repolarization (Fig. 5B), due to inactivation of rapid I_{to} . APD prolongation observed at later phases of repolarization was consistent with a previous study of genetically altered guinea pig myocytes.³⁸ Differences in I_{to} between the monolayers, constructs, and ventricles may be due to differences in the number of cell-to-cell contacts, which was shown to positively correlate with $Kv4.2$ mRNA expression,⁴¹ and/or possible

differences in the percentage of fibroblasts, which was shown to inversely correlate with Kv4.2 mRNA expression.³⁷

In native ventricles, prolongation of the APD by 4-AP was dose dependent (Fig. 6A). Although previous studies showed that 4 mM 4-AP completely blocked the I_{to} in single cardiac myocytes,^{25,28} it is likely that cells within 3-D preparations, such as tissue constructs and native ventricles, exhibit higher resistance to drugs as previously shown for other cell types.^{3,42} At a concentration of 12 mM, 4-AP prolonged the APD₂₀, APD₅₀, and APD₉₀ of ventricles to match the corresponding APDs and MCRs measured for baseline (drug-free) tissue constructs (Fig. 6A and B). Together, these data imply that decreased I_{to} is the major cause of APD prolongation in tissue constructs as compared with ventricles, and that stimulation during cell refractoriness is the underlying cause of blocked propagation in constructs stimulated at rates higher than the MCR.

Alternatively, APD prolongation in monolayers and tissue constructs as compared with neonatal ventricles could be due to either slowed I_{Na} inactivation or increased I_{Ca-L} density. The former possibility is unlikely, on the basis of a previous report of faster and not slower I_{Na} inactivation in cultured cat myocytes,⁴³ while an increase in I_{Ca-L} density was previously shown to contribute to APD prolongation in monolayers.^{40,44}

In the present study, action potentials were recorded via “blindly” positioned microelectrodes that could conceivably have impaled a nonmyocytic cell within the sample. We observed only small differences in nonpacemaker action potential shapes throughout a single sample, which can be explained by the fact that fibroblastic cells are tightly coupled via gap junctions to adjacent cardiomyocytes *in vitro* and *in vivo*,^{23,45,46} such that the two cell types are expected to exhibit almost identical action potential characteristics.⁴⁵ In the present study, tissue constructs were generated from cells obtained from neonatal ventricles by enzymatic dissociation followed by preplating, a method expected to yield >80% myocytic cells, but also other components including endothelial cells and fibroblasts.⁴ Use of a mixed cell population may have mimicked some aspects of the milieu present during native cardiogenesis, for example, signaling by direct cell-to-cell contact and via soluble mediators (e.g., Conderelli *et al.*⁴⁷).

Although the feasibility of cardiac tissue engineering has been demonstrated *in vitro* (e.g., Eschenhagen *et al.*⁸) and *in vivo* (e.g., Li *et al.*¹⁸), further increases in construct size and functionality are needed.^{12,48} In particular, the lack of an interconnected capillary network and associated oxygen transport limitations currently limits the thickness of cardiac tissue constructs to ~0.1 mm.⁷ We reported that the interstitial flow of culture medium enabled the *in vitro* cultivation of constructs with signif-

icantly higher cell viabilities and cell densities than nonperfused controls.²¹ In more recent *in vivo* studies, circular constructs fit around the circumference of hearts survived, matured, and remained contractile for several weeks postimplantation in syngeneic rats,¹⁹ and layered cardiomyocyte sheets remained contractile for up to 12 weeks after subcutaneous implantation in nude rats.²⁰ One active area of ongoing research involves defining appropriate cell sources for human cardiac repair; possible candidates include autologous cells, or allogeneic cells that are either rendered nonimmunogenic or used in conjunction with immunosuppressants.

In summary, neonatal rat cardiomyocytes cultured for 7 days in 3-D tissue constructs maintained an *in vivo*-like phenotype better than the same cells cultured in confluent monolayers, as evidenced by levels of Cx-43, CK-MM, and MHC, and cell-scale electrophysiological properties. With structural and functional differences in mind, tissue constructs and monolayers can provide complementary model systems for *in vitro* developmental, electrophysiological, and pharmacological studies of cardiac muscle; with further progress, implanted tissue constructs could eventually provide a means of improving the electromechanical function of damaged cardiac muscle.

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