Constitutive Properties of Adult Mammalian Cardiac Muscle Cells

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Background—The purpose of this study was to determine whether changes in the constitutive properties of the cardiac muscle cell play a causative role in the development of diastolic dysfunction.

Methods and Results—Cardiocytes from normal and pressure-hypertrophied cats were embedded in an agarose gel, placed on a stretching device, and subjected to a change in stress (σ), and resultant changes in cell strain (ε) were measured. These measurements were used to examine the passive elastic spring, viscous damping, and myofilament activation. The passive elastic spring was assessed in protocol A by increasing the σ on the agarose gel at a constant rate to define the cardiocyte σ-versus-ε relationship. Viscous damping was assessed in protocol B from the loop area between the cardiocyte σ-versus-ε relationship during an increase and then a decrease in σ. In both protocols, myofilament activation was minimized by a reduction in [Ca2+]. Myofilament activation effects were assessed in protocol C by defining cardiocyte σ versus ε during an increase in σ with physiological [Ca2+]. In protocol A, the cardiocyte σ-versus-ε relationship was similar in normal and hypertrophied cells. In protocol B, the loop area was greater in hypertrophied than normal cardiocytes. In protocol C, the σ-versus-ε relation in hypertrophied cardiocytes was shifted to the left compared with normal cells.

Conclusions—Changes in viscous damping and myofilament activation in combination may cause pressure-hypertrophied cardiocytes to resist changes in shape during diastole and contribute to diastolic dysfunction. (Circulation. 1998;98:567-579.)

Key Words: hypertrophy ■ myocytes ■ diastole ■ elasticity ■ heart failure

Congestive heart failure can be caused by a primary abnormality in systolic function, a primary abnormality in diastolic function, or both. The basic mechanisms causing diastolic CHF are not fully understood. We and others have hypothesized that changes in both the extracellular matrix and the cardiac muscle cell are causally responsible for the changes in diastolic function that occur during the development of diastolic CHF. To date, however, even the most basic questions about the role played by the cardiocyte in the development of diastolic CHF have not been addressed. For example: (1) Are cardiocyte constitutive properties such as stiffness and viscosity altered in diastolic CHF? and (2) What cellular structures or processes cause any changes in cardiocyte constitutive properties? Before these and other questions could be addressed, methods for measuring cardiocyte constitutive properties had to be developed and validated. A variety of techniques have been proposed, but each had specific limitations in the context of the pathophysiological questions we wished to address. Therefore, the first purpose of this study was to validate a new technique (the “gel-stretch method”) for examining cardiocyte constitutive properties.

The second purpose of this study originated in both clinical and experimental studies showing that cardiac hypertrophy induced by chronic hemodynamic overloading causes significant changes in ventricular and myocardial diastolic function and the development of CHF. We and others have hypothesized that changes in cardiocyte constitutive properties are causally responsible for the changes in diastolic function. This hypothesis is based on studies suggesting that myocardial resistance to deformation during the application of stress cannot be explained by the stiffness of the extracellular matrix alone. Thus, this resistance to deformation must be dependent on structures intrinsic to the cardiocyte. Over a physiological range of sarcomere lengths, elements responsible for most of the passive force in cardiac muscle clearly reside within the myofibril. To date, however, whether cardiocyte constitutive properties are altered by chronic hemodynamic overloading has not been well defined. Therefore, the second purpose of this study was to use our new technique to determine whether chronic pressure-overload hypertrophy alters the constitutive properties of isolated cardiocytes.
The third purpose of this study was to determine what changes in cellular structures or processes might alter cardiocyte constitutive properties. It was postulated that changes in both myofilament and nonmyofilament structures and processes, such as the extramyofilament cytoskeleton and calcium homeostasis, might affect cardiocyte constitutive properties. To date, however, no studies have determined whether changes in the cytoskeleton affect stiffness or viscosity at the cellular level. A number of factors make it likely that changes in one cytoskeletal structure, the microtubules, could play an important role in altering cardiocyte stiffness and viscosity. Microtubules form along stress axes and bear intracellular and transcellular loads. Pressure-overload hypertrophy, which increases ventricular and myocardial stiffness, has been shown to increase microtubule density, whereas other components of the extramyofilament cytoskeleton do not appear to be altered. In contrast, volume-overload hypertrophy, which does not increase ventricular or myocardial stiffness, is associated with normal microtubule density. Microtubules themselves are known to have viscoelastic properties and may play a significant role in providing viscoelastic resistance to a deforming stress. Therefore, we hypothesized that changes in cardiocyte constitutive properties may be caused by a change in the extramyofilament cytoskeleton or calcium homeostasis.

Methods

Pressure- and Volume-Overload Hypertrophy
RVPOH was created by PAB. RVVOH was created by an ASD. RVH was documented by hemodynamic studies. Cardiocytes were enzymatically isolated.

Gel-Stretch Method

Gel Preparation
After isolation, cardiocytes were added to a solution composed of 2% agarose, HEPES-Krebs buffer, and lammin. This suspension was then poured into molds. Each gel contained 100 000 to 250 000 cardiocytes. The gel was extracted from the mold, immersed in HEPES-Krebs buffer, placed in an incubator at 37°C, and bubbled with oxygen. To study cardiocytes, the gel was mounted on the mandibles of the test apparatus; the gel sample itself and the mandibles that held it were immersed in a chamber filled with HEPES-Krebs buffer placed on a movable inverted microscope stage directly over the objective. The cross-sectional area of the sample in the area of cardiocyte observation was 2.1×10⁻⁵ m².

Test Apparatus
The gel sample was held between an adjustable roller and a fixed plate that acted together as a mandible (Figure 1). The mandibles were connected to separate ball-screw assemblies arranged in parallel and connected to each other by nylon gears. The stepper motor turned one threaded shaft, the second threaded shaft turned an equal amount in the opposite direction. The stepper motor operated at 240 steps per revolution, creating a displacement in the screw shafts of 1 mm per rotation. The stepper motor was controlled by a custom-designed electronic circuit developed for this application. The load applied to the gel was measured with a load cell (model 31, Synsotec) positioned on the left mandible arm so that the mandible had a 2:1 mechanical advantage. The indicated load was converted to gel stress according to the following computation: stress (σ) = force/area. σ = (L)(gf)(g)(kg/1000 g)/(G-CSA)(233.57), where gf is a geometric factor (0.5), L is load in grams, g is acceleration due to gravity (9.81 m/s²), and G-CSA is cross-sectional area of the gel sample (0.000021 m²). Stress applied to the gel did not equal the stress on the cardiocyte within the gel. Stress on the cardiocyte was calculated by use of the finite-element analysis described below. Strain was calculated by imaging cardiocytes at variable loads with an inverted microscope with a ×40 Hoffman modulation contrast objective. Nominal strain (ε) = (Lₙ − Lᵢ)/Lᵢ, where Lᵢ is initial length and Lₙ is new length obtained after stretch.
Stretch Protocol
Load on the gel was increased at a rate of 1 g/min. This resulted in a strain rate in the gel of \( \approx 10 \, \text{μm/min} \). This increased cardiocyte stress at a rate of \( \approx 1 \, \text{kN} \cdot \text{m}^{-2} \cdot \text{min}^{-1} \) and cardiocyte strain at \( \approx 0.1 \, \text{μm/min} \). Stretch was performed under load control rather than length control, because length values required manual measurement and were not available on line. Only those cells whose long axis was parallel to the direction of stretch were studied.

Cardiocyte Constitutive Properties

Measurement of Cardiocyte Stress
Data derived from the gel-stretch method included measurements of stress on the gel and strain in the cardiocyte. In addition, it was necessary to measure stress on the cardiocyte itself. The three steps necessary to measure stress on the cardiocyte were (1) define the material properties of the agarose gel itself, (2) develop an FEM to describe cardiocyte constitutive properties, and (3) calculate cardiocyte stress from experimental data using the FEM-determined cardiocyte constitutive properties.

Mechanical Properties of the Agarose Gel
Tensile tests were performed to determine the material properties of the agarose gel. A polynomial relationship of the form \( \sigma = C_1 e + C_2 e^2 \) was assumed for the mechanical behavior of the gel. The constants \( C_1 \) and \( C_2 \) were determined by fitting the experimental data to the polynomial curves by a least-squares analysis. By use of these methods, a constitutive equation for the agarose gel was derived.

Finite-Element Model
We assumed that there was a single cell embedded in an infinitely large gel medium within a cylinder of 150-μm radius and 1400-μm height. An axisymmetric model was used for a single plane of the cell. Both the gel and cell were modeled as hyperelastic incompressible materials with a displacement of 60 μm. Boundary conditions for both the cell and gel were applied along the lines of symmetry. Convergence and error analysis were used to determine the appropriate number of elements, which approximated 9000. Material properties for the system were defined from the constitutive equations for the agarose gel (\( \sigma = 51 \, \text{kN/m}^2 \cdot e + 343 \, \text{kN/m}^2 \cdot e^2 \)) and the cardiocyte (\( \sigma = C_1 e + C_2 e^2 \)). A uniaxial tensile displacement in the longitudinal direction was applied in increments to the cardiocyte-gel system. An initial guess for the cardiocyte properties (\( C_1, C_2 \)) was made on the basis of experimental data (gel stress versus cardiocyte strain) and an elastic analysis of the agarose gel system. The predicted values of both longitudinal and lateral cardiocyte strain from the FEM were compared with the experimentally observed values. Constants \( C_1 \) and \( C_2 \) were adjusted accordingly until the strain values from the model matched the strain values from the experiments. Once this iterative process was complete, the newly determined constitutive equation was used to plot cardiocyte stress versus cardiocyte strain for each set of experimental data.

Determinants of the Cardiocyte Stress-Versus-Strain Relationship
The slope and position of the cardiocyte stress-versus-strain curve are affected by three major determinants: the passive elastic spring, the viscous damping constant, and myofilament activation (Figure 2). The passive spring consists of all the cellular elements that resist stretch in a time-independent manner. To calculate differences in passive spring properties between two populations of cells, the rate at which force is increased (and thus the displacement rate) must be constant and slow. Furthermore, the level of myofilament activation must be at or near zero (protocol A). Damping elements consist of the cellular structures or processes that resist stretch in a time-dependent manner, i.e., they resist more when stretched faster. Differences in viscous damping between two populations of cardiocytes can be determined by altering calcium levels by methods such as altering the buffer calcium concentration (protocol C). Therefore, the methods used to generate the cardiocyte stress-versus-strain relationship dictate which of these three determinants are examined and which constitutive cardiocyte properties are characterized.

Experimental Protocols
Cardiocytes were isolated from 20 normal cats, 15 cats with RVPOH for 2 weeks, and 3 cats with RVVVOH for 2 weeks. In each animal, an average of 5 to 8 RV cardiocytes were studied. Cardiocytes isolated from normal, 5 PAB, and 3 ASD cats underwent protocols A and B, cardiocytes from normal and 5 PAB cats underwent protocol C, and cardiocytes from 10 normal and 5 PAB cats underwent protocol D.

Protocol A
The passive elastic spring was assessed by application of force to the agarose gel at a constant rate of 1 g/min. To reduce myofilament activation to a minimum, cardiocytes were treated with 7 mmol/L BDM, 0.1 mmol/L EGTA, and 0 mmol/L added calcium. Cardiocyte images were recorded at 5-g intervals from 0 to 40 g. Data were plotted initially as gel stress versus cardiocyte strain, then replotted with the FEM as cardiocyte stress versus cardiocyte strain. Changes in cardiocyte passive elastic spring properties were measured as \( d\sigma/d\varepsilon \) at 1%, 5%, and 8% strain, the energy imparted to the cardiocyte during an increase in force, and the constants of three exponential equations: \( \sigma = A e^{\alpha t} \), \( \sigma = A (e^{\alpha t} - 1) \), and \( \sigma = A/k(e^{\beta t} - 1) \). We hypothesized that if RVH made the cardiocyte passive spring less elastic, the cardiocyte stress-versus-strain relationship would shift to the left (Figure 3).

The energy (E) imparted to the cardiocyte during an increase in stress was assessed by calculating the integral of force applied to the cardiocyte and the distance that the cardiocyte moved as a result of this force application (Figure 4B). Distance was equal to the difference between the initial length (L<sub>i</sub>) and the length after the application of
stress ($L_i$). This energy was calculated by assessing the integral area (Area) under the cardiocyte stress–versus–cardiocyte strain curve during an increase in stress (Figure 4C) and was calculated as $E \left( \frac{N \cdot m}{m^2} \right) = \text{Area} \left( \frac{N/m^2}{m^2} \right) \cdot \text{CSA} \left( m^2 \right) \cdot \text{L}_i \left( m \right)$ (Figure 4D). The average cross-sectional area of the cell was 2865 $\mu m^2$. The average distance was 10.88 $\mu m$. The average initial length was 136 $\mu m$.

Protocol B

Viscous damping was assessed by calculating the loop area between the stress-versus-strain relationship during a sequential increase then decrease in stress. When stress is increased, the area within the stress-strain relationship is equal to the potential energy gained by the cell during the application of force (Figure 5A). When stress is decreased, the energy returned to the system is equal to the integral of stress and strain (Figure 5B).

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**Figure 3.** Schematic showing hypotheses tested in this study.

**Figure 4.** Schematic describing methods used in protocol A to assess changes in passive elastic spring properties. See text for details.

**Figure 5.** Schematic describing “loop area method” used in protocol B to assess presence and extent of viscous damping. See text for details.
If there is damping, there will be a difference between the energy gained during the application of a stress and the energy returned when this stress is decreased (Figure 5C). This loop area between these two stress-strain relationships represents the energy lost to heat and reflects the amount of viscous damping within a system (Figure 5D). The loop area, expressed in arbitrary units, was determined initially by plotting gel stress versus cardiocyte strain. Then, by the FEM, data were replotted as cardiocyte stress versus cardiocyte strain, with loop area expressed as kN/m². Stress was increased at a rate of 1 g/min from 0 to 40 g and then decreased at a rate of 1 g/min from 40 to 0 g. Cardiocyte images were captured at 5-g intervals. Cardiocytes were treated with BDM, EGTA, and no added calcium. We hypothesized that if viscous damping were increased, the loop area would also increase (Figure 3).

**Protocol C**
Change in the level of myofilament activation was examined in normal and PAB cardiocytes under protocol C. In protocols A and B, the level of myofilament activation was kept at a minimum by treating cells with BDM, EGTA, and 0 mmol/L added calcium. In protocol C, BDM and EGTA were omitted from the protocol, and cardiocytes were studied in solutions containing 2.5 mmol/L calcium. Data obtained during this protocol were analyzed by the same methods as those described in protocol A. As shown in Figure 3, we hypothesized that if myofilament activation were abnormal, the stress-versus-strain relationship in normal calcium would be shifted toward the left.

**Protocol D**
The effects of altering microtubule density on the passive elastic spring and viscous damping were measured in normal and PAB cardiocytes by treating them with colchicine or taxol and subjecting them to protocols A and B. The effects of acute microtubule depolymerization were examined by treating cardiocytes from 5 normal and 5 PAB cats with 10⁻⁵ mol/L colchicine for 60 minutes. Colchicine causes microtubule depolymerization by preventing α,β-tubulin heterodimers from polymerizing into microtubules. That is, because microtubules are in dynamic equilibrium between polymerized microtubules and α,β-tubulin heterodimers, and the half-life of a microtubule is ~30 minutes, if polymerization is prevented, the number of microtubules will steadily decrease as microtubules spontaneously depolymerize and new microtubules are prevented from forming. The effects of acute microtubule hyperpolymerization were examined by treating cardiocytes from 5 normal cats with 10⁻⁵ mol/L taxol for 60 minutes. Taxol, by stabilizing microtubules, increases microtubule density. Cardiocytes were treated with colchicine or taxol before they were embedded in the agarose gel; however, both the agarose gel and the medium that superfused the agarose gel had the same concentration of colchicine or taxol. Thus, throughout the study period, these colchicine or taxol effects were maintained. The effects on microtubule polymerization of treating cardiocytes with colchicine or taxol has been examined extensively in our previous studies by use of both immunoblots to quantify the relative amounts of free and polymerized tubulin and confocal immunofluorescence micrographs to examine the appearance and density of the microtubule network.

**Cardiocyte Adhesion Studies**
One experimental variable that in and of itself might cause differences in measured cardiocyte properties is the affinity of normal and PAB cardiocytes for attachment to laminin. The affinity of cardiacocytes for the extracellular matrix protein ligands laminin and fibronectin was examined in normal and PAB cardiocytes. In 3 PAB cats, cardiocytes were isolated from the hypertrophied RV and the normal left ventricle. Multiwell dishes were precoated with either laminin or fibronectin at multiple concentrations, and cardiocytes were plated at a concentration of 150,000 cells/well and allowed to attach for 60 minutes. Unattached cells were removed by gentle washing of the wells. The number of attached cardiocytes was then determined.

**Data Analysis and Statistics**
Measurements obtained from RVH and normal cats were compared by an unpaired t-test. Data for the cardiocytes from a given animal were averaged. The mean data for a group of animals were derived from these average values. Variation in cardiocyte properties within groups of cardiocytes is presented in Figures 6, 8, and 9. Figures 6 and 9 display gel stress-versus-cardiocyte strain data from protocols A and C as mean strain ± SEM for each 5-g increment in stress during an increase in stress from 0 to 40 g. Stress values do not have standard errors because stretches were done under load (not length) control. For protocols B and D, data are presented in Figure 8 as the mean loop area ± SEM. Differences between group means for assessment of cardiocyte constitutive properties by measurements derived from protocols A through D, considered significant at P<0.05, were determined with a multway ANOVA and a Newman-Keuls multiple sample comparison test.

**Results**

**Effects of RVH**
Measurements of pressure, oximetry, and mass are summarized in Table 1. Cardiocyte size and sarcomere length at baseline, ie, before stretch at a resting force of 0 kN/m², are summarized in Table 1. Data are similar to those in previous studies.7,36 PAB caused significant RVPOH. ASD caused a significant RVVOH and RVH that was comparable to that caused by PAB.

**Determinants of the Cardiocyte Stress-Versus-Strain Relationship**

**Protocol A: Passive Elastic Spring**

**RVPOH**
Group data examining gel stress versus cardiocyte strain and cardiocyte stress versus cardiocyte strain in normal and PAB cardiocytes are shown in Figure 6. This relationship in both groups of cardiocytes was curvilinear. At ~10 kN/m², there was an equivalent degree of cardiocyte strain in both groups (8±1 in normal versus 8±1 in PAB). Figure 6 also shows the gel stress-versus-sarcomere strain relation for normal cardiocytes and PAB cardiocytes. This relationship was nearly identical in these two groups of cardiocytes. The mean values for cardiocyte stress-versus-cardiocyte strain by the FEM during an increase in stress were similar in a normal cardiocyte to those in a PAB cardiocyte. From this relationship, dσ/dε at 0.01, 0.03, and 0.08 strain, as well as the area under the stress-versus-strain curve, the energy gained by the cardiocyte during the increase in stress, and the constants A and k in the exponential equations given above, were determined. These data are presented in Table 2. There were no significant differences between normal and PAB cardiocytes with respect to any of these measures.

**RVVOH**
The cardiocyte stress-versus-strain relationship during an increase in stress was similar in normal and ASD cardiocytes. There were no significant differences between normal and ASD in dσ/dε at 0.01, 0.03, and 0.08 strain, the area under the stress-strain curve, the energy gained by the cardiocyte, and the constants A and k for the exponential equations. In ASD cardiocytes, A was 25 kN/m² and k was 13. Thus, there were no significant differences in the stress-versus-strain relationship between normal, PAB, and ASD cardiocytes, suggesting that the passive elastic spring was not changed during RVPOH or RVVOH.
Protocol B: Viscous Damping

RVPOH

An example of the gel stress–versus–cardiocyte strain relationship in a normal cardiocyte during an increase and decrease in force is shown in Figure 7. There was a clear finite-loop area between these lines, which reflects damping. This loop area was greatly increased in PAB cardiocytes. An example of cardiocyte stress versus cardiocyte strain by use of the FEM in normal and PAB cardiocytes during a sequential increase and decrease in stress is shown in Figure 7. There was a marked increase in the loop area in the PAB compared with the normal cardiocytes. In addition, the stress-versus-strain relationship during a decrease in stress was distinctly different in the normal compared with the PAB cardiocytes. This relationship was shifted downward in the PAB compared with the normal cardiocytes. The constants (A and k) that describe this relationship were significantly different in the normal compared with PAB cardiocytes (Table 2). Summary data for groups of normal and PAB cardiocytes are shown in Figure 8. These data were derived from the gel stress–versus–cardiocyte strain relationship during a sequential increase and decrease in stress. In normal cardiocytes studied in the baseline state (normal-baseline), the average loop area of 0.46 kN/m² was distinctly smaller than the average loop area in the PAB cardiocytes studied in the baseline state (hypertrophy-baseline) of 0.87 kN/m². The larger loop area in the PAB cardiocytes suggested an increase in viscous damping in PAB cardiocytes.
The average loop area in the ASD cardiocytes (0.43 kN/m²) was not significantly different from that in normal cardiocytes. Thus, cardiocyte viscous damping was increased by RVPOH but was unchanged by RVVOH.

### TABLE 1. Effects of RV Hypertrophy on Hemodynamics and Cell Size

<table>
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<tr>
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<th>Normal</th>
<th>PAB</th>
<th>ASD</th>
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<tr>
<td><strong>In vivo hemodynamic studies</strong></td>
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<tr>
<td>RV systolic pressure, mm Hg</td>
<td>24±1</td>
<td>63±5*</td>
<td>25±3</td>
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<tr>
<td>ΔO₂% saturation, SVC vs RA</td>
<td>−0.5±0.3</td>
<td>−0.2±0.5</td>
<td>11.5±4.2*</td>
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<td>RV wt/body wt, g/kg</td>
<td>0.55±0.04</td>
<td>0.96±0.06*</td>
<td>0.86±0.11*</td>
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<td>RV wt/tibial length, g/cm</td>
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<td>0.20±0.01*</td>
<td>0.21±0.06*</td>
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<td>LV wt/body wt, g/kg</td>
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<td>2.1±0.1</td>
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<td>Body wt, kg</td>
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<td>3.5±0.6</td>
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<tr>
<td>Arteriovenous O₂ difference, mL/L</td>
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<td>34±2</td>
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<tr>
<td>RV diastolic pressure, mm Hg</td>
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<td>4±1</td>
<td>1±3</td>
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<tr>
<td>Liver wt/body wt, g/kg</td>
<td>33±1</td>
<td>30±1</td>
<td>27±4</td>
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<table>
<thead>
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<th>ASD</th>
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<tr>
<td><strong>In vitro morphometric studies</strong></td>
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<td>Cardiocyte diameter, mm</td>
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<tr>
<td>Cardiocyte area, mm²</td>
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<td>3345±98*</td>
<td>3251±137*</td>
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<td>Cardiocyte length, mm</td>
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<td>Sarcomere length, μm</td>
<td>1.87±0.01</td>
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<td>1.86±0.05</td>
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LV indicates left ventricular; SVC, superior vena cava; and RA, right atrium. *P<0.05 vs normal.

**RVVOH**

The average loop area in the ASD cardiocytes (0.43 kN/m²) was not significantly different from that in normal cardiocytes. Thus, cardiocyte viscous damping was increased by RVPOH but was unchanged by RVVOH.

### TABLE 2. Cardiocyte Constitutive Properties

<table>
<thead>
<tr>
<th></th>
<th>Protocol A*</th>
<th>Protocol B†</th>
<th>Protocol C*</th>
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<tr>
<td></td>
<td>Normal</td>
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<td>Normal</td>
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<tr>
<td>C₁ (kN/m²)</td>
<td>188</td>
<td>267</td>
<td>300</td>
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<tr>
<td>C₂ (kN/m²)</td>
<td>3452</td>
<td>1916</td>
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<tr>
<td>C₃ (kN/m²)</td>
<td>180 000</td>
<td>217 230</td>
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<tr>
<td>dσ/dε, kN/m²</td>
<td>1%</td>
<td>223</td>
<td>116</td>
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<td></td>
<td>5%</td>
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<td>8%</td>
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<td>Area, kN/m²</td>
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<tr>
<td>Energy, Nm</td>
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<td>6.7×10⁻¹⁰</td>
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<tr>
<td>Loop area, kN/m²‡</td>
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<td>0.87</td>
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<tr>
<td>Loop energy, Nm‡</td>
<td>1.79×10⁻¹⁰</td>
<td>4.33×10⁻¹⁰</td>
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</tr>
</tbody>
</table>

**Equation 1**

A, kN/m²  | 0.50  | 275  | 0.25  | 0.012 | 0.75  | 515  |
| k        | 60    | 10   | 66    | 70    | 50    | 14.5 |

**Equation 2**

A, kN/m²  | 17    | 16   | 0.18  | 0.03  | 14.5  | 35   |
| k        | 14.5  | 14.5 | 69    | 85    | 14.5  | 14.5 |

**Equation 3**

A, kN/m²  | 23.0  | 27.5 | 2.3   | 0.4   | 29.5  | 51.5 |
| k        | 16    | 10   | 60    | 80    | 6.5   | 14.5 |

*σ indicates cardiocyte stress; ε, cardiocyte strain; C₁ and C₂, constants in the polynomial equation σ=C₁ε+C₂ε²; and A and k, constants in the exponential equations relating stress and strain. Equation 1, σ=Aε⁶; Equation 2, σ=A(ε⁶−ε); Equation 3, σ=Ak(e³−1). *

*Data derived from σ vs ε during an increase in σ; †data derived from σ vs ε during a decrease in σ; and ‡data derived from loop area between σ vs ε during an increase in σ and σ vs ε during a decrease in σ.*
calcium concentrations of 2.5 mmol/L was shifted to the left and was steeper than the gel stress–versus–cell strain relationship in PAB cardiocytes treated with BDM, EGTA, and no added Ca\(^{2+}\). The mean values for cardiocyte stress–versus–cardiocyte strain with the FEM and protocol C are shown in Figure 9. The stress-versus-strain relationship in the PAB cardiocytes studied in physiological calcium was shifted to the left compared with PAB cardiocytes treated with BDM, EGTA, and no added calcium. In contrast, the stress-versus-strain relationship in normal cardiocytes was unchanged by the presence of physiological calcium. Consequently, these data suggest that changes in the level of myofilament activation occurred during the development of RVPOH and increased cardiocyte stiffness.

Protocol D: Effects of Altering Microtubule Polymerization
Cardiocytes from normal and PAB cats were treated with 10^{-6} mol/L colchicine for 60 minutes (Figure 8). Colchicine did not affect the slope or position of the stress-versus-strain relationship during an increase in force in either normal or PAB cardiocytes. Colchicine caused only a small decrease in the loop area of normal cardiocytes (0.30 kN/m²) but caused a large and significant decrease in the loop area of PAB cardiocytes (0.27 kN/m²). In particular, colchicine caused the loop area in the PAB cardiocytes to decrease to a value

![Figure 7. Data derived from protocol B. Top, Example of gel stress–vs–cell strain relationship during increase in stress (solid symbols) and a decrease in stress (open symbols) in a normal cardiocyte (circles) is compared with that in an RVPOH cardiocyte created by PAB (triangles). In both cardiocytes, there is a clear finite area between stress-vs-strain curves, indicating presence of viscous damping; however, loop area is significantly larger in pressure-hypertrophied cardiocyte. Bottom, With FEM, cardiocyte stress–vs–cardiocyte strain relationships were assessed. Loop area for pressure-hypertrophied cardiocytes is much larger than that for normal cardiocytes. Therefore, RVPOH increases viscous damping.](http://circ.ahajournals.org/)

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was responsible, in large part, for the increased damping normal cardiocytes but did increase viscous damping. Thus, taxol did not change the passive elastic spring in normal or PAB cardiocytes in the baseline state.

Colchicine caused a small decrease in loop area of normal cardiocytes (normal - colchicine) and caused a large and significant decrease in loop area of hypertrophied cardiocytes (hypertrophy - colchicine). Normal cardiocytes were treated with 10^{-5} mol/L colchicine for 60 minutes. Colchicine caused a small decrease in loop area of normal cardiocytes (normal - colchicine) and caused a large and significant decrease in loop area of hypertrophied cardiocytes (hypertrophy - colchicine). Normal cardiocytes were treated with 10^{-5} mol/L taxol for 4 hours, which caused a significant increase in their loop area (normal - taxol).

Figure 8. Group data examining effects of RVPOH and microtubule polymerization state on cardiocyte viscous damping. Loop area reflects damping and is presented in arbitrary units. Compared with group of normal cardiocytes studied in control state (normal - baseline), average loop area in group of RVPOH cardiocytes studied in control state (hypertrophy - baseline) was significantly increased. Cardiocytes from normal and RVPOH cats were treated with 10^{-6} mol/L colchicine for 60 minutes. Colchicine caused a small decrease in loop area of normal cardiocytes (normal - colchicine) and caused a large and significant decrease in loop area of hypertrophied cardiocytes (hypertrophy - colchicine). Normal cardiocytes were treated with 10^{-5} mol/L taxol for 4 hours, which caused a significant increase in their loop area (normal - taxol).

The data obtained in the present study suggest that (1) RVPOH does not alter the properties of the passive elastic spring; (2) RVPOH causes an increase in viscous damping, which itself is caused by an increase in the microtubule portion of the cardiocyte cytoskeleton; and (3) RVPOH causes an alteration in the level of myofilament activation. Thus, in chronic pressure-overload hypertrophy, changes in viscous damping and myofilament activation combine to cause an increase in the resistance to cardiocyte shape changes. In contrast, volume overload has no effects on the presence of physiological levels of calcium. Cardiocytes can be stretched over a physiological length range without undergoing plastic, irreversible changes; they return to rest length after stretch; and morphology as well as sarcomere definition and resting length are unchanged.

Data from the present study using the gel-stretch technique are concordant with studies done in isolated mammalian cardiocytes using a variety of other techniques. Granzier and Irving and Brady examined the stress-versus-strain relationship in normal cardiocytes. Their range of stresses and sarcomere lengths and their values of A and k were similar to those found in the present study. LeGuennec et al and Fish et al also studied cardiocytes over a similar range of sarcomere lengths; however, these investigators required significantly lower stress values to obtain these sarcomere lengths. Consequently, measured values of k were lower than those in the present study. It is possible that these differences may be based in part on the state of intracellular titin in these preparations. Nonetheless, taken as a whole, the cardiocyte data presented in the present study have many parallels with data from other studies using very different techniques.

The gel-stretch method provided certain advantages. It allowed selective assessment of the passive elastic spring, viscous damping, and myofilament activation, which was not possible with previous techniques. It did not require the use of detergent-skinned preparations, a process that in and of itself may alter the constitutive properties of cardiocytes. The sarcolemma was protected from potentially harmful effects of direct cardiocyte attachment. The gel-stretch method did not require direct cardiocyte attachment to a force or length transducer, and the force was applied along the entire cardiocyte length. Cardiocytes embedded in agarose remain mechanically and morphologically intact. In addition, we believe that the gel-stretch method reproduced the in vivo setting more closely than other techniques. Studies have shown that the constitutive properties of cells are contact-dependent. Cardiocytes embedded in agarose are surrounded by a matrix that influences the entire surface of the cardiocyte rather than just the portion of the cardiocyte attached at two ends. Within the agarose gel, cardiocytes are calcium-tolerant, and sarcolemmal membranes remain intact, all of which recreates more closely the environment under which the cardiocyte lengthens in vivo. There is evidence that intracellular calcium concentration and calcium homeostasis may alter viscoelastic properties of isolated cardiocytes. Thus, the ability to study cardiocytes in medium containing physiological calcium concentrations is a major advantage of the gel-stretch method.

**Effects of Pressure and Volume Overload**

The data obtained in the present study suggest that (1) RVPOH does not alter the properties of the passive elastic spring; (2) RVPOH causes an increase in viscous damping, which itself is caused by an increase in the microtubule portion of the cardiocyte cytoskeleton; and (3) RVPOH causes an alteration in the level of myofilament activation. Thus, in chronic pressure-overload hypertrophy, changes in viscous damping and myofilament activation combine to cause an increase in the resistance to cardiocyte shape changes. In contrast, volume overload has no effects on the

**Cell Adhesion Study**

Adhesion of normal and hypertrophied cardiocytes to laminin and fibronectin was comparable at all protein concentrations tested, up to a saturating level of each (Figure 10).

**Discussion**

**Gel-Stretch Method**

The gel-stretch method provides accurate measurements of cardiocyte constitutive properties in normal and hemodynamic-overload states. With this technique, a definable stress applied to the cardiocyte results in a measurable change in cardiocyte and sarcomere strain. Substantial numbers of cardiocytes from each animal can be studied, they do not need to be skinned before study, and they can be studied in the
cardiocyte constitutive properties. The comparison of these two models of chronic overload hypertrophy is important and unique, for the following reasons. Both result in substantial and similar extents of hypertrophy in the RV. Therefore, their effects on cardiocyte constitutive properties are independent of the presence of hypertrophy and specific to the type of hemodynamic overload present. The constitutive properties of cardiocytes isolated from these two models parallels the results obtained from analysis of their myocardial properties. Chronic RVPOH is characterized by significant abnormalities in passive diastolic stiffness and active diastolic relaxation and a significant increase in microtubule density.22,47,48 It might be expected, therefore, that cardiocyte constitutive properties in this type of hemodynamic overload would be abnormal. In contrast, chronic RVVOH does not alter passive stiffness or active relaxation and does not increase microtubule density.22,47,48 Therefore, cardiocyte constitutive properties would be expected to be normal in this type of hemodynamic overload.

Data presented in this study using the agarose stretch method were concordant with data obtained from two other independent techniques that we previously used to examine several aspects of the viscoelastic properties of isolated cardiocytes: osmolar stress and magnetic twisting cytometry.6–8 Kato et al7 used the osmolar stress technique to study cardiocytes from RVPOH and RVVOH cats. In the osmolar stress technique, changes in the osmolality of the superfusate buffer created a force (stress) that altered the size and shape
The viscosity of the extramyofilament cytoskeleton affects cardiocyte shape, size, and stiffness in vivo. Microtubule depolymerization with colchicine reduced cytoskeletal viscosity in RVPOH cardiocytes to normal. Conversely, microtubule hyperpolymerization by taxol increased cytoskeletal viscosity in normal cardiocytes to a value comparable to that of hypertrophied RV cardiocytes.

The stiffness of the cardiocyte cytoskeleton was also measured by magnetic twisting cytometry. When cardiocytes were studied in physiological calcium, there was a significant increase in the passive elastic spring of the cardiocyte cytoskeleton in the RVPOH cardiocytes compared with normal cardiocytes. However, when RVPOH cardiocytes were treated with BDM, EGTA, and no added calcium, stiffness decreased significantly to a value that approached but did not reach normal. At first glance, these data may appear contradictory with the gel-stretch data, which suggested that there was no change in the passive spring of the RVPOH cardiocyte as a whole, whereas the magnetic twisting cytometry data suggested that the passive spring of the cytoskeleton was increased in RVPOH. However, changes in cytoskeletal stiffness or any single component of the cardiocyte may or may not predict changes in overall cardiocyte stiffness, because the cardiac muscle cell should be modeled as a composite material, and the overall stiffness of a composite material is proportional to, or most affected by, the stiffness of the element(s) within the composite having the greatest stiffness. To measure the stiffness of a composite material, the material as a whole must be deformed, ie, a known force must be applied to the material and a resultant deformational strain must be measured. Magnetic twisting cytometry does not measure the composite material properties of the cardiocyte as a whole because it does not deform the cardiocyte. Instead, the magnetic bead selectively attaches to an integrin, producing only local shear stress during rotation, and does not deform the cardiocyte or the sarcomeres. Rather, it deforms only those cytoskeletal proteins that are attached to the integrin. Thus, magnetic twisting cytometry selectively measures the material properties of the cytoskeleton, one single component of the composite, but does not examine the material properties of the cardiocyte, the composite as a whole. Although it is easy to envision how an increase in microtubule network density would alter viscosity, it is unlikely that the extramyofilament cytoskeleton would have a stiffness that exceeds that of the myofilaments themselves. It is also easy to envision that the stiffness of the cardiocyte as a whole would be primarily dependent on the myofilaments.

**Clinical Applications**

In vivo, cardiocytes lengthen at a physiological rate of about 10 μm/s in the presence of physiological levels of calcium. Thus, in vivo, cardiocytes lengthen at a rate that would engage viscous damping forces and at a time when myofilament activation is more than nominal. Therefore, when cardiocytes lengthen under conditions that parallel the in vivo physiological condition, all 3 determinants, ie, the passive elastic spring, viscous damping, and myofilament activation, may affect the resistance to changes in cardiocyte shape, even (strain) of the cardiocyte. The resultant osmolarity-versus-strain relationship was then used to examine relative changes in cardiocyte properties. When this experiment was done in the presence of EGTA and no added calcium, changes in the osmolarity-versus-strain relationship were determined principally by changes in the passive elastic spring. Kato et al showed that the position, shape, and steepness of the osmolarity-versus-strain relationship were the same in normal cardiocytes and in RV hypertrophied cardiocytes from both PAB and ASD cats. Thus, neither RV volume overload nor RV pressure overload altered the passive elastic spring. The osmolar stress technique was limited in its ability to characterize cardiocyte constitutive properties, however, because the long-axis cardiocyte strain changed by <4%, cardiocyte viscosity could not be measured, cardiocytes were not studied in the presence of physiological calcium, and cardiocyte stress could not be quantified. These limitations were in part responsible for the need to develop the gel-stretch method.

Tagawa et al and others used the magnetic twisting cytometry technique to study cardiocytes from RV pressure-overload cats produced by PAB. For the magnetic twisting cytometry technique, RGD-coated ferromagnetic beads were attached to the cardiocyte sarcolemmal integrins. These beads were then twisted by application of a perpendicular magnetic field. Because the beads were attached through cell surface integrins to the tensegrity network of the cytoskeleton, the twist applied a rotational force (stress) to the microtubules that resulted in their deformation (strain). This method did not affect cardiocyte shape or size and did not produce cardiocyte deformation or cardiocyte strain. Therefore, this method directly examined the properties of the cytoskeleton and not the properties of the cardiocyte as a whole. These studies showed that the viscosity of the extramyofilament cytoskel-
in normal cardiocytes. If pathological conditions such as chronic pressure-overload hypertrophy alter the amount of viscous damping, change the level of myofilament activation, or alter the passive elastic spring, this would further affect the resistance to cardiocyte shape change. Therefore, a change in any of these three determinants either alone or in concert would result in an alteration in the cardiocyte stress-versus-strain relationship when the cardiocyte was studied under conditions that parallel the in vivo physiological state. Before cardiocytes could be studied under conditions that parallel the in vivo physiological state, it was important first to isolate the effect of each determinant on the cardiocyte stress-versus-strain relationship and to determine whether any of these three determinants were altered by chronic pressure-overload hypertrophy. To do this, each determinant was examined in isolation with the other two held constant and only one allowed to vary at a time. This was the principal goal of the present study. Future studies should be aimed at studying cardiocytes under conditions that parallel the in vivo physiologic state and examine the cardiocyte stress-versus-strain relationship at a time when all three determinants are changing simultaneously. These kinds of studies would allow assessment of the relative importance of each determinant to the cardiocyte stress-versus-strain relationship in vivo and allow assessment of the composite stress-versus-strain relationship. Such studies, however, must await the technical ability to impose a force or length change at a physiological rate and to measure the cardiocyte stress and strain at that rate.

Summary
This study shows that the gel-stretch technique provides accurate measurements of cardiocyte constitutive properties. RVPOH does not alter the cardiocyte passive elastic spring. RVPOH causes an increase in cardiocyte viscous damping, which in turn is caused by an increased density of the microtubule portion of the cardiocyte cytoskeleton. RVPOH causes an alteration in the level of myofilament activation. RVVHOH does not alter cardiocyte constitutive properties. Thus, in RVPOH, changes in viscous damping and myofilament activation cause an increase in the resistance of the cardiocyte to changes in shape.

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