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# Pulsatile Stretch Remodels Cell-to-Cell Communication in Cultured Myocytes

Jianping Zhuang, Kathryn A. Yamada, Jeffrey E. Saffitz, André G. Kléber

**Abstract**—Mechanical stretch is thought to play an important role in remodeling atrial and ventricular myocardium and may produce substrates that promote arrhythmogenesis. In the present work, neonatal rat ventricular myocytes were cultured for 4 days as confluent monolayers on thin silicone membranes and then subjected to linear pulsatile stretch for up to 6 hours. Action potential upstrokes and propagation velocity ( $\Theta$ ) were measured with multisite optical recording of transmembrane voltage of the cells stained with the voltage-sensitive dye RH237. Expression of the gap junction protein connexin43 (Cx43) and the fascia adherens junction protein N-cadherin was measured immunohistochemically in the same preparations. Pulsatile stretch caused dramatic upregulation of intercellular junction proteins after only 1 hour and a further increase after 6 hours (Cx43 signal increased from 0.73 to 1.86 and 2.02% cell area, and N-cadherin signal increased from 1.21 to 2.11 and 2.74% cell area after 1 and 6 hours, respectively). This was paralleled by an increase in  $\Theta$  from 27 to 35 cm/s after 1 hour and 37 cm/s after 6 hours. No significant change in the upstroke velocity of the action potential or cell size was observed. Increased  $\Theta$  and protein expression were not reversible after 24 hours of relaxation. Nonpulsatile (static) stretch produced qualitatively similar but significantly smaller changes than pulsatile stretch. Thus, pulsatile linear stretch in vitro causes marked upregulation of proteins that form electrical and mechanical junctions, as well as a concomitant increase in propagation velocity. These changes may contribute to arrhythmogenesis in myocardium exposed to acute stretch. (*Circ Res.* 2000;87:316-322.)

**Key Words:** remodeling ■ stretch ■ connexin43 ■ conduction velocity

Cardiac hypertrophy and failure are known to interfere with normal electrical function, and the associated increased incidence of sudden death is related to ventricular arrhythmias.<sup>1-3</sup> Several mechanisms may underlie altered electrical function and arrhythmogenesis such as ectopic impulse generation due to early and delayed afterdepolarizations and circus movement re-entry due to disturbances in impulse conduction and heterogeneity in repolarization.<sup>4-6</sup> Altered cell-to-cell coupling in hypertrophy and failure is predicted to have an impact on both heterogeneous repolarization and impulse conduction. The proteins involved in electrical and metabolic cell-to-cell communication appear to be affected in a complex way by hypertrophy and failure. Early mediators of myocardial hypertrophy, such as cAMP<sup>7</sup> and angiotensin II,<sup>8</sup> have been shown to increase cell-to-cell coupling in cultured myocytes within 24 hours, which suggests that cell-to-cell electrical coupling might be upregulated in early stages of hypertrophy. Early upregulation of cardiac gap junction proteins was indeed observed in renovascular hypertension.<sup>9</sup> In later stages of experimental and clinical cardiac failure, electrical cell-to-cell coupling has been shown to decrease.<sup>9-11</sup>

Mechanical stretch is thought to play an important role in the remodeling of the cardiac phenotype, and a number of studies have characterized the response of cultured myocytes to mechanical stretch. For example, static 10% stretch of randomly oriented neonatal rat myocytes increases proto-oncogene and contractile protein expression and stimulates signaling pathways, including those involving tyrosine kinases, Ras/mitogen-activated protein (MAP) kinase pathways, protein kinase C, and phospholipases C and D.<sup>12-15</sup> Recent studies by Seko et al<sup>16</sup> have demonstrated that pulsatile stretch (PS) activates all 3 MAP kinase family members by mechanisms mediated, in part, by autocrine release of vascular endothelial growth factor and transforming growth factor- $\beta$ . Because many of the responses to mechanical stretch by neonatal rat cardiac myocytes in vitro seem to recapitulate features of the hypertrophic response, in vitro stretch appears to be a good model of cardiac responses to overload in vivo.

Our laboratory has developed a technique for growing myocytes on substrates that are accessible for multiple-site optical recording of transmembrane potential.<sup>17</sup> In the present study, this technique was applied to cells grown on transpar-

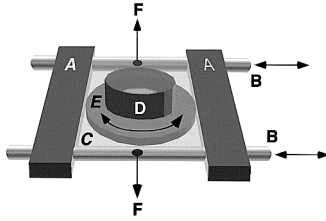
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**Figure 1.** Schematic presentation of the custom-fabricated stretch apparatus. Horizontal metal bars (A) glide horizontally on stainless cylindrical axes (B) and support the transparent silicone membrane (C). A segment of silicone tubing (D) was glued on the silicone membrane to form the walls of the culture dish in which the neonatal rat myocytes were seeded and grown. One full rotation of the elliptical wheel E (ratio of longitudinal to transverse diameter of 1.1:1) produced 2 stretch cycles of 10% of initial length. A photocell (not shown) controlled the frequency of stretch. Two clamps (F; positions indicated by vertical arrows) produced slight tension along the central axis of the stretch apparatus and thereby reduced transverse shrinking to <1%.

ent silicon membranes (kindly provided by Dr Frank Yin, Washington University, St. Louis, Mo). In this way, the effects of acute PS on transmembrane action potentials and conduction velocity were compared with changes in expression of the major cardiac gap junction protein connexin43 (Cx43) and the fascia adherens junction protein N-cadherin in the same preparations. Our results indicate that PS induces marked upregulation in expression of proteins responsible for electrical and mechanical cell-to-cell coupling with a concomitant increase in conduction velocity within 1 hour.

## Materials and Methods

### Stretch Apparatus

The custom-designed and custom-fabricated device used for the application of linear PS on cultured cardiac myocytes is illustrated in Figure 1. The lateral borders of a rectangular sheet of silicone membrane (thickness 0.01 inch) were fixed to Teflon bars that could move freely in the *x* direction along 2 stainless steel axes. The 2 bars were in contact with an elliptical Teflon wheel mounted in the center of the device. The silicone membrane was cut to a length that produced tension slightly above the slack length when the short diameter of the wheel was in contact with the Teflon bars. The 1.1/1 ratio of the long to the short diameter of the elliptical wheel produced stretch of 10% during a 90° rotation of the wheel. The frequency of the stretch pulsations (half a wheel cycle) was monitored by a photodiode mounted on the stretch apparatus and controlled from outside the tissue culture incubator. A stretch frequency of 3 Hz was used in the present experiments. Transverse compression (shrinkage) in the *y* direction during linear stretch in the *x* direction was prevented by attaching 2 clamps that stretched the membranes slightly along the transverse central axis. Cells were cultured in a chamber created by fixing a ring of silicone tubing (thickness 2 mm, diameter 24 mm) at the center of the membrane with silicone sealant. Uniformity of stretch in the culture area was determined in the presence of the silicone ring during stretch periods of up to 72 hours and amounted to  $10 \pm 1\%$ . Transverse deformation of the membrane at maximal longitudinal stretch was <1%. Hence, stretch was uniaxial.

### Cell Cultures

Primary cultures of 1- to 2-day-old neonatal rat ventricular myocytes were prepared as reported previously.<sup>17</sup> Cells were cultured in M199 (GIBCO) supplemented with penicillin (20 U/mL), streptomycin (20  $\mu$ g/mL), vitamin B12 (2  $\mu$ g/mL), and 10% neonatal calf serum. The

cell suspension was preplated to reduce the fibroblast content. Epinephrine (0.01  $\mu$ mol in 1-mL cell suspension) was added to the medium during the first 72 hours of culture. The silicone membranes were coated with collagen IV<sup>18</sup> before cell seeding to ensure adhesion of the cells to the membrane and growth of dense cultures devoid of large intercellular clefts. Cells were grown in a random orientation (so-called isotropic growth<sup>18</sup>) and kept in an incubator at 35°C in a humidified atmosphere containing 0.07% CO<sub>2</sub>.

### Optical Mapping and Analysis of Propagation

The technique of multiple-site optical recording of transmembrane potential and the staining of cell cultures with the voltage-sensitive dye RH237 have been described in detail elsewhere.<sup>19</sup> The cultures were stimulated at a site located >1 mm from the recording site, and isochronal maps were calculated as previously described.<sup>20</sup>

### Experimental Procedure

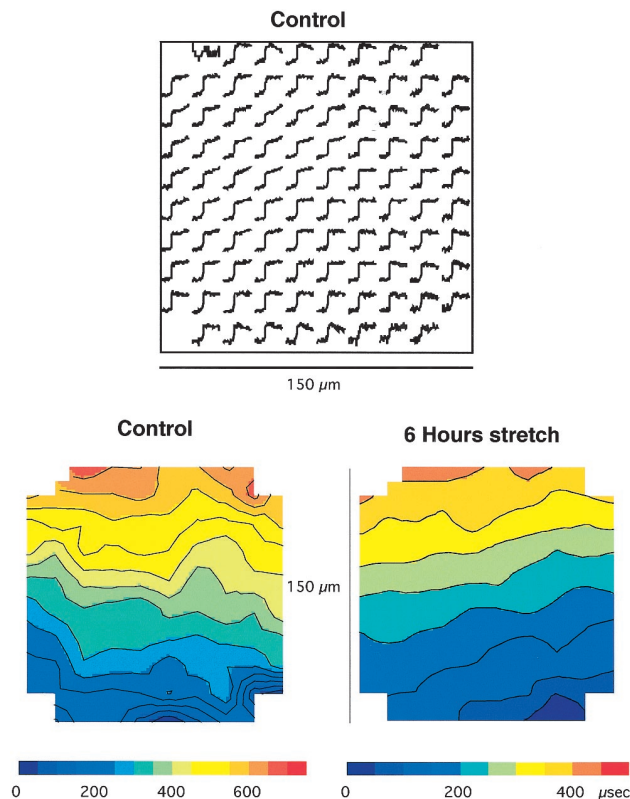
After seeding and preplating, cultures were incubated for 4 days. Thereafter, the culture medium was replaced with medium containing 5% FCS every 24 to 48 hours. Cultures were then subjected to controlled pulsatile linear stretch for test periods of 1, 3, or 6 hours. Each series of experiments was performed on 8 to 12 cultures derived from 1 cell suspension. In each series, half of the cultures were exposed to test conditions, and the other half served as controls. After each test period, the silicone membranes were stabilized in the 0%-stretch position within a metal frame, removed from the stretch apparatus, and brought to the stage of an inverted microscope for optical mapping. The specific sites of optical mapping analysis in each membrane were noted by placing a small notch at one edge of the membrane and devising an (*x*, *y*) coordinate system, with the notch representing the top of the *y* axis. In each case, most of the optical measurements were made within a single quadrant within 3×3 mm of the center of the membrane. At the completion of optical mapping analysis, the cultures were processed for immunohistochemistry. In some series, all cultures were stretched for 6 hours. Half of the cultures were then fixed and analyzed by immunohistochemistry, whereas the other half were allowed to recover from stretch and were analyzed 24 hours later. Several control experiments were carried out. First, PS was compared with nonpulsatile stretch for a period of 6 hours. In a second series, the spontaneous changes in electrical activity and expression levels of intercellular junction proteins occurring between 4 and 5 days of culture were determined.

### Immunohistochemistry

Myocytes on silicone membranes were fixed in 4% paraformaldehyde in PBS for 15 minutes and rinsed 3 times in PBS. Each membrane was cut into quadrants using the notch as a guide. The quadrant (3×3 mm) containing all or most of the optical recording sites was immunostained using an affinity-purified polyclonal rabbit anti-Cx43 antiserum (Zymed) diluted 1:200 in a blocking buffer composed of PBS containing 0.1% Triton X-100, 3% normal goat serum, and 1% BSA. Another quadrant containing all or most of the remaining optical recording sites was immunostained with a polyclonal rabbit antiserum against a conserved sequence in the N-cadherins (Sigma) diluted 1:400 in blocking buffer. All immunostaining procedures, including the use of controls for nonspecific binding, have been described in detail in a previous report.<sup>21</sup> Immunostained cells were mounted on glass slides and examined with a Sarastro model 2000 laser scanning confocal microscope (Molecular Dynamics).

### Confocal Microscopy

Clusters of myocytes immunostained with antibodies against either Cx43 or N-cadherins were identified in each membrane, corresponding to the approximate locations of previous multiple site optical recordings of transmembrane potential. Five high-power fields in each membrane were examined by fluorescence microscopy at a magnification of ×400 as previously described.<sup>21</sup> The proportion of



**Figure 2.** Top, Transmembrane action potential upstrokes recorded from 96 sites (corresponding to a field of  $150 \times 150 \mu\text{m}$ ) in a control culture. Bottom left, Isochronal map calculated from a control culture on top panel. Bottom right, Isochronal map calculated after 6 hours of pulsatile horizontal stretch. Isochronal lines are drawn at intervals of  $50 \mu\text{s}$ . Stretch direction is horizontal.

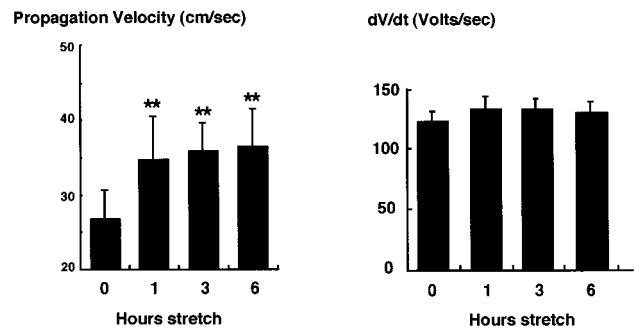
total cell area occupied by Cx43 or N-cadherin immunoreactive signal was defined as the number of high signal-intensity pixels divided by the total number of pixels occupied by cells. The total number and mean size of individual spots of high-intensity signal, operationally defined as individual gap junctions or fascia adherens junctions in cells stained with anti-Cx43 or anti-N-cadherin antibodies, respectively, was measured according to methods described and validated in previous studies.<sup>21</sup> The 5 individual values for percentage of cell area occupied by immunoreactive signal, as well as the number and size of gap junctions and fascia adherens junctions, were averaged to yield single values of each parameter for each membrane.

### Measurement of Cell and Nuclear Size

A quadrant of the silicone membrane not used for immunostaining was analyzed by light microscopy in selected control and stretched preparations to determine whether PS led to changes in cell or nuclear size. The cells were stained with 1% toluidine blue/methylene blue. In each culture, 2 fields of myocytes, similar to those analyzed by optical mapping and immunohistochemistry in other quadrants of the same membrane, were photographed at a final print magnification of  $\times 800$ . The areas of individual cells and nuclei were measured using computer-assisted planimetry and averaged for each culture.

### Statistical Analysis

Data are expressed as mean  $\pm$  SD. Optical mapping data, confocal microscopy data, and measurements of cell and nuclear areas were analyzed by 1-way ANOVA (SigmaStat). The nonpaired Student *t*



**Figure 3.** Group data for propagation velocity (left) and upstroke velocity (right). Mean  $\pm$  SD values are shown for control cells and cells subjected to 1, 3, or 6 hours of PS. \* $P < 0.01$  vs control (significant differences).

test was used to compare data obtained at a given test condition with control data.  $P \leq 0.05$  was considered significant.

## Results

### Effect of PS on the Transmembrane Action Potential Upstroke and Propagation Velocity

PS was associated with a marked and rapid effect on electrical activity. The results recorded in 4 culture dishes from the same batch are illustrated in Figure 2. The top panel of Figure 2 shows the transmembrane action potential upstrokes recorded before PS from 96 sites. The corresponding isochronal map is depicted in the bottom left panel. The bottom right panel illustrates an isochronal map calculated after 6 hours of PS. Mean conduction velocity,  $\Theta$ , and mean values of the maximal upstroke velocities  $(dV/dt)_{\text{max}}$  in each field of vision were calculated from 8 to 10 randomly selected sites, and the values for each culture dish were calculated from the means determined in 4 to 5 fields of vision. As illustrated by the increase in the spacing of the isochrones in Figure 2 and the data shown in Figure 3 and Table 1, propagation velocity increased significantly from 27 cm/s (control) to 35 cm/s (1-hour PS) and 37 cm/s (6-hour PS). The control value for  $\Theta$  of 27 cm/s in the isotropic cultures corresponded to the 25 cm/s previously reported from cell cultures grown on glass substrates.<sup>18</sup> The upstroke velocity of the action potential,  $(dV/dt)_{\text{max}}$ , increased slightly from 126% action potential amplitude (APA)/ms (corresponding to V/s at an APA of 100 mV<sup>17</sup>) to 134% APA/ms during the 6 hours of PS. No dependence of propagation on the direction of stretch could be detected. Control experiments involving up to 72 hours of PS and comparison of propagation parallel and perpendicular to the stretch axis revealed purely isotropic propagation (J. Zhuang and A.G. Kléber, personal communication, April 2000).

### Effect of PS on Cx43 Expression

Representative confocal images of Cx43 immunohistochemical staining are shown in Figure 4, and group data are shown in Table 1 (top) and Figure 5. As observed in previous studies, gap junctions in cultured neonatal rat ventricular myocytes are distributed in a "neonatal" pattern characterized by a regular distribution of small dotlike junctions around the cell perimeter.<sup>20</sup> This pattern was confirmed in the present



**TABLE 1. Remodeling of Conduction Velocity, Action Potential Upstroke Velocity, Cx43, and N-Cadherin Proteins by PS**

|                                      | Action Potential<br>Upstroke<br>Velocity, % APA<br>per ms | n  | Conduction<br>Velocity,<br>cm/s | n  | Gap Junctions,<br>No. per Field of<br>Vision | n  | Relative Gap<br>Junction Size | n  | Cx43 Protein, %<br>Cell Area | n  | N-Cadherin<br>Protein, %<br>Area | n  |
|--------------------------------------|---|----|---------------------------------|----|--|----|-------------------------------|----|------------------------------|----|----------------------------------|----|
| Control Versus PS                    |   |    |                                 |    |  |    |                               |    |                              |    |                                  |    |
| Control                              | 126±12  | 9  | 27±4                            | 9  | 1288±373                                     | 9  | 0.57±0.10                     | 9  | 0.73±0.16                    | 9  | 1.21±0.15                        | 9  |
| 1-hour PS                            | 138±11 (NS)   | 9  | 35±6*                           | 9  | 2801±117*                                    | 9  | 0.69±0.12                     | 9  | 1.86±0.71*                   | 9  | 2.11±0.22*                       | 9  |
| 3-hour PS                            | 138±11 (NS)   | 7  | 36±4*                           | 7  | 2588±871*                                    | 7  | 0.71±0.11                     | 7  | 1.76±0.43*                   | 7  | 2.24±0.13*                       | 7  |
| 6-hour PS                            | 134±12 (NS)   | 11 | 37±5*                           | 11 | 2792±625*                                    | 11 | 0.75±0.10†                    | 11 | 2.02±0.29*                   | 11 | 2.74±0.42*                       | 11 |
| PS Versus PS Plus Relaxation         |   |    |                                 |    |  |    |                               |    |                              |    |                                  |    |
| 6-hour PS (control)                  | 129±9   | 4  | 35±2                            | 4  | 2494±467 (NS)                                | 4  | 0.78±0.16 (NS)                | 4  | 1.81±0.17 (NS)               | 4  |                                  |    |
| 6-hour PS plus<br>24-hour relaxation | 126±14  | 4  | 35±2                            | 4  | 2450±35 (NS)                                 | 4  | 0.83±0.03 (NS)                | 4  | 1.96±0.07 (NS)               | 4  |                                  |    |

No. of culture dishes (4 to 9 fields of vision were analyzed per dish).

\* $P < 0.01$ .

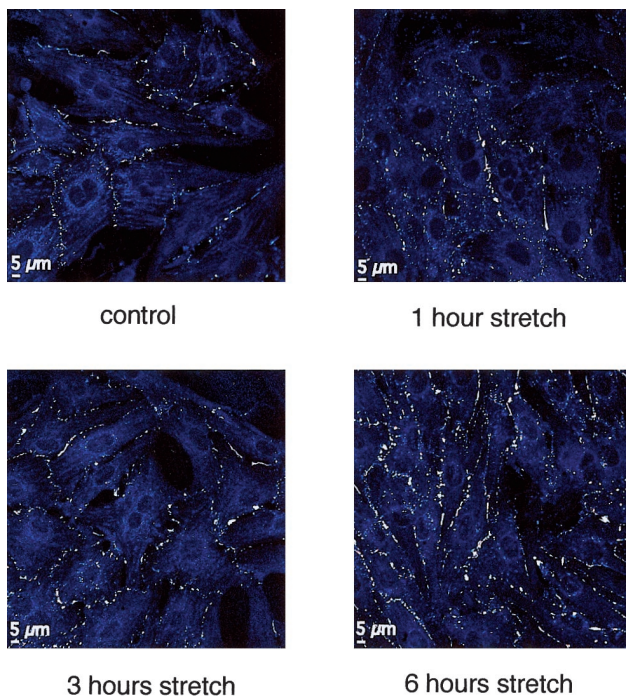
† $P < 0.05$ .

experiments and did not change during 6 hours of directed PS. However, a significant and marked increase in the amount of Cx43 immunoreactive signal was observed between control and 1 hour of PS and continued for up to 6 hours of PS. Cx43 signal increased by  $\approx 3$ -fold after 6 hours. This was due mainly to an increase in the number of discrete spots of signal in the confocal images, which were operationally defined as individual gap junctions. In contrast, the mean size of an individual gap junction increased only slightly and reached statistical significance only after 6 hours of PS (Table 1, top). Control experiments comparing  $\Theta$ ,  $(dV/dt)_{max}$ , and Cx43 signal in cells grown in culture for 4 or 5 days (without

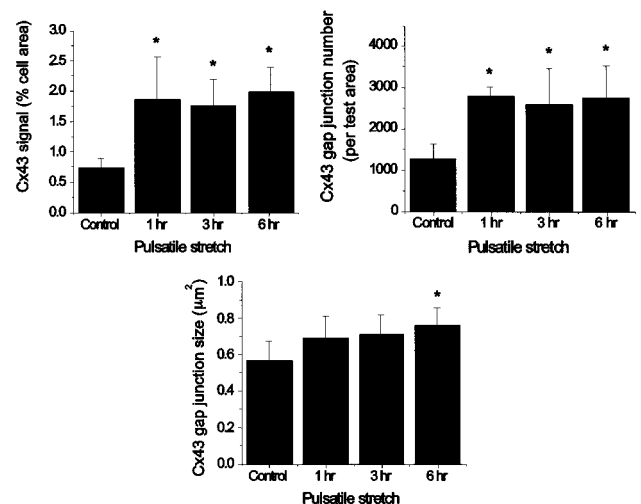
PS) revealed no differences. This demonstrated that no stretch-independent changes in the above parameters occurred during this stage of growth.

#### Effect of PS on N-Cadherin Expression and Cell Size

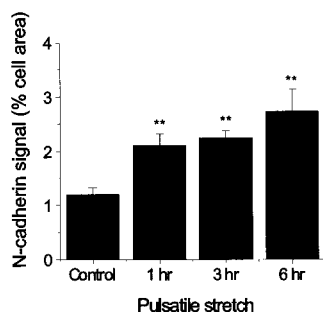
It has been shown recently that formation of new cell-to-cell junctions in myocyte cultures typically involves initial formation of mechanical junctions after which gap junctions containing connexins appear.<sup>22</sup> In the present experiments, the question whether mechanical and electrical junctions would be affected by stretch in a similar way was addressed. As shown in Figure 6, the amount of N-cadherin-immunoreactive signal was increased in cells subjected to PS with a



**Figure 4.** Representative confocal images of Cx43 immunofluorescent signal distribution in cell monolayers. Top left, Control; top right, 1-hour PS; bottom left, 3-hour PS; bottom right, 6-hour PS.



**Figure 5.** Group data of quantitative confocal analysis of Cx43 signal in control cells and cells subjected to 1, 3, or 6 hours of PS. Top left, Cx43 signal (% cell area). Top right, Cx43 gap junction number. Bottom: Cx43 gap junction size ( $\mu\text{m}^2$ ). \* $P < 0.01$  vs control (statistically significant differences). Note marked increases in Cx43 signal due almost entirely to an increase in gap junction number. Gap junction size increased only modestly during stretch and became significant only after 6 hours.



**Figure 6.** Group data of quantitative confocal analysis of N-cadherin signal (% cell area) in control cells and cells subjected to 1, 3, or 6 hours of PS. \* $P < 0.01$  vs control (statistically significant differences).

time course similar to that seen for Cx43 signal. Double immunolabeling showed an intimate spatial relationship between sites of electrical and mechanical junctions that were closely interspersed in regions of the junctional membrane without dependence of upregulation on stretch direction.

Light microscopic measurements of cell and nuclear size revealed no differences between control cultures and cultures subjected to PS for 6 hours (Table 2). However, comparison of the angle between the longest cell axis and the direction of stretch showed a slight cell alignment after 6 hours of PS ( $53 \pm 21^\circ$  in control versus  $39 \pm 20^\circ$ ,  $n = 108$ ;  $P < 0.001$ ).

### Reversibility of Stretch-Induced Changes and Effect of Pulsatile Versus Nonpulsatile Stretch

Cultured cells were subjected to either 6 hours of PS or 6 hours of PS followed by 24 hours of relaxation. As shown in Table 1, bottom, changes in conduction velocity, Cx43 immunoreactive signal, and gap junction number caused by 6 hours of PS were not significantly reversed after 24 hours of relaxation.

Nonpulsatile stretch, produced by turning the elliptic wheel (Figure 1)  $90^\circ$  from its rest position to a steady-state position causing static 10% stretch, changed both propagation velocity and Cx43 immunoreactive signal. After 6 hours, propagation velocity increased from  $30 \pm 3$  to  $34 \pm 2$  cm/s, and Cx43 immunoreactive signal from  $0.94 \pm 0.03\%$  to  $1.59 \pm 0.03\%$  cell area ( $n = 7$ ;  $P < 0.01$ ). Thus, the changes seen with static stretch were smaller than those caused by an equivalent interval of PS.

### Discussion

Our results indicate that linear PS of 10% resting length produced marked upregulation of Cx43 and N-cadherin in intercellular junctions within 1 hour. A further increase was observed after 6 hours. This upregulation corresponded to an increase in propagation velocity, measured in the same preparations. This indicates that most of the gap junction protein newly integrated into the cell membrane contributed to cell-to-cell transmission of electrical current. Recently, upregulation of Cx43 protein determined by Western blotting was reported after only 4 hours of 20% PS.<sup>23</sup> Changes in intercellular connections in hypertrophy and failure, condi-

**TABLE 2. Cell Area and Cell Nuclear Size**

|                | Cell Area, $\mu\text{m}^2$ | Nuclear Area, $\mu\text{m}^2$ |
|----------------|----------------------------|-------------------------------|
| Control        | $825 \pm 63$               | $116 \pm 6$                   |
| 1-hour stretch | $737 \pm 144$              | $115 \pm 13$                  |
| 3-hour stretch | $982 \pm 184$              | $130 \pm 8$                   |
| 6-hour stretch | $771 \pm 62$               | $119 \pm 12$                  |

Changes shown are not significant.

tions associated with increased ventricular mechanical load, appear to be complex. In the early stages of ventricular hypertrophy, results obtained from cell cultures have shown upregulation of Cx43 expression and gap junctions induced by early mediators of hypertrophy, such as cAMP<sup>7</sup> and angiotensin II.<sup>8</sup> In the late stage of cardiac failure, conduction velocity and most likely connexin expression are downregulated.<sup>10</sup>

The techniques used in these studies allowed (1) measurement of changes in proteins forming both mechanical and electrical junctions and (2) direct association of the molecular findings with changes in function. The relationship between formation of mechanical and electrical junctions is of potential importance, because it was recently shown that reestablishment of cell-to-cell contacts requires formation of mechanical junctions before electrical junctions can be assembled.<sup>22</sup> In accordance with these results, our data show a close association between regulation of the 2 types of junctions during mechanical stretch. The question of whether concomitant changes in different types of cell-to-cell contacts are regulated by a common signaling pathway awaits further investigation.

Although upregulation of Cx43 expression and a marked increase in gap junction number were prominent features of short-term stretch, other mechanisms could have contributed to the observed increase in propagation velocity. A first factor relates to upregulation of other connexins. Indeed, upregulation of Cx45 (protein and mRNA) was shown to follow application of dibutyryl cAMP concomitantly with Cx43. Whether upregulation of Cx45 or other connexins occurs with PS remains to be shown. A second factor, which is likely to play a role after long-term stimulation and which was ruled out in the present experiments, is a change in cell size and shape. An increase in cell size would be expected to increase conduction velocity.<sup>24</sup> Although a small alignment of cells was observed after 6 hours of PS, even prolonged stretch up to 72 hours did not induce electrical anisotropy in the present experiments. A third potential factor relates to the possible upregulation of  $\text{Na}^+$  channels carrying the main electrical charge during the action potential upstroke. Although a contribution of enhanced  $\text{Na}^+$  channel activity to our results cannot be ruled out, the lack of a significant change in upstroke velocity during short-term PS suggests that this contribution was not substantial.

A semiquantitative relationship between the resistance of the intracellular space of a cellular network,  $r_i$ , and propagation velocity,  $\Theta$ , in electrically continuous tissue is given by  $\Theta^2 \sim 1/r_i$ .<sup>24,25</sup> In this relationship, the sum of cytoplasmic and gap junctional resistances is the main resistive element

determining conduction velocity.<sup>19,24</sup> If it is assumed that the increase in Cx43 immunoreactive signal is proportional to the increase in gap junctional conductance, a 2.5-fold increase in signal after 1-hour stretch in Cx43 signal would lead to a 27% increase in  $\Theta$ , and a 3-fold increase would correspond to a 32% increase. These theoretically estimated increases in conduction velocity are close to the experimentally determined values of 27% and 39%, respectively. A similar close correlation was observed in cultured myocytes in which upregulation of Cx43 was induced by dibutyl cAMP.<sup>7</sup> The observations in the present study suggest that most of the immunoreactive Cx43 was present in electrically functional gap junctions and any contribution of a change in depolarizing  $I_{Na}$  was less important.

Two additional observations regarding the stretch effects are worth noting. First, the changes induced by PS were only slightly reversible after 24 hours of relaxation. Second, static stretch of the same amount (10%) and duration (6 hours) caused qualitatively similar but quantitatively smaller changes. The reversibility of stretch-induced changes has not been studied systematically, although Yamazaki et al<sup>26</sup> have reported that activation of MAP kinase activity in cultured myocytes subjected to 20% static stretch is greater after 2 minutes of stretch than after 2 minutes of stretch and 6 minutes of relaxation. Increasing evidence suggests that pulsatile and static stretch may activate different signaling pathways. For example, activation of extracellular signal-regulated protein kinase (ERK) 1/ERK2 by PS appears to be mediated, in part, by vascular endothelial growth factor and transforming growth factor- $\beta$  but not by angiotensin II or endothelin.<sup>16</sup> In contrast, autocrine release of angiotensin II and endothelin mediates activation of intracellular signaling in myocytes subjected to static stretch.<sup>27,28</sup> The signaling pathways mediating the effects of PS on expression of intercellular junction proteins have not been defined. Nor is it clear whether accumulation of protein in intercellular junctions was due to increased synthesis, decreased degradation, enhanced translocation of intracellular protein to cell surface junctions, or a combination of these mechanisms. It was recently reported, however, that Cx43 mRNA levels increased after 4 hours of 20% PS.<sup>23</sup>

Differences observed in the magnitude of changes induced by pulsatile and static stretch in the present study might be related to the superimposition of PS at 3 Hz on spontaneous electrical excitation and/or to modulation of background spontaneous electrical activity. A recent report indicates that incorporation of [<sup>3</sup>H]leucine and activation of p44/42 MAP kinase and phosphorylation of its upstream activator MAP/ERK1 and -2 were significantly greater in cultured myocytes subjected to 4% stretch during systole than during diastole, whereas stretch-induced activation of p38 MAP kinase and c-Jun NH<sub>2</sub>-terminal protein kinase (JNK) did not depend on the timing of stretch with respect to the cardiac cycle.<sup>29</sup>

In summary, our results demonstrate that PS induces rapid changes in expression of proteins responsible for electrical and mechanical cell-to-cell communication associated with a marked increase in impulse propagation velocity. An increase in electrical cell-to-cell coupling can increase the discontin-

uous nature of propagation and thereby contribute to formation of unidirectional block and arrhythmia initiation.<sup>30</sup>

### Acknowledgments

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