Cyclic Mechanical Stretch Induces Cardiomyocyte Orientation and Polarization of the Gap Junction Protein Connexin43

Aida Salameh, Anne Wustmann, Sebastian Karl, Katja Blanke, Daniel Apel, Diana Rojas-Gomez, Heike Franke, Friedrich W. Mohr, Jan Janousek, Stefan Dhein

Rationale: Cyclic mechanical stretch (CMS) is an important physiological and pathological factor in the heart.
Objective: We examined whether CMS can affect localization of gap junctions with regard to the cell axis.
Methods and Results: Neonatal rat cardiomyocytes were cultured (7 days) on flexible 6-well plates. Thereafter, cells were kept static or stimulated with CMS (1 Hz; 0, 10, 20% elongation) for 0, 24, or 48 hours (with or without 10 μmol/L PD98059, 5 μmol/L BIM I (bisindolylmaleimide I), 2 μmol/L H8 [N-(2-methylamino-ethyl)-5-isoquinoline-sulfonamid], or 0.1 μmol/L angiotensin II. Additionally, cells were exposed to 24 hours of CMS followed by 24 hours of static recovery. CMS (24 hour, 10%) induced elongation of the cardiomyocytes and orientation 79°±8° toward the stretch direction. Moreover, the distribution of connexin (Cx)43 together with N-cadherin changed, so that both proteins were accentuated at the cell poles, whereas in nonstretched cells, they were distributed around the cell without preferential localization. Additional angiotensin II reduced polar Cx43 accentuation. The CMS-induced changes in Cx43 were reversible within 24 hours after end of stretch, and could be completely prevented by the MEK1/2 inhibitor PD98059 but not by BIM I or H8. Moreover, stretch resulted in Cx43 protein and Cx43-mRNA upregulation and in a significant upregulation of the phosphorylated forms of ERK1/2, glycogen synthase kinase 3β and AKT. Furthermore, CMS resulted in a significant increase of the transcription factors activator protein 1 and CREB (cAMP response element-binding protein) in the nucleus.
Conclusions: CMS results in self-organization of cardiomyocytes leading to elongated cells oriented transverse to the stretch axis, enhanced Cx43 expression and Cx43 accentuation at the cell poles. The Cx43-changes seem to depend on the ERK1/2 signaling cascade. (Circ Res. 2010;106:1592-1602.)

Key Words: cyclic mechanical stretch ■ cardiomyocytes ■ connexin43 ■ N-cadherin ■ ERK ■ angiotensin

Intercellular communication is an important feature of organization within many types of tissue. Gap junction channels form the basis of direct intercellular communication. The structure of these important channels was discovered by Unwin and Zampighi in 19801 and their model of a gap junction channel holds true until today: the whole pipe-like gap junction channel is composed of 2 hemichannels, the so-called connexons, each being composed of 6 protein subunits, the connexins. A connexin consists of 4 transmembrane domains, 2 extracellular and 1 intracellular loop, as well as the N and C terminus at the cytoplasmic side of the cell. The C terminus is the most variant part of a connexin and differs in length and amino acid sequence between the various connexin isoforms. The C terminus contains various consensus sequences for a number of protein kinases, thereby regulating connexin turnover.2-5 In human heart it has been shown that connexin (Cx)43, which has a considerably short half-life of ~90 minutes, is the predominantly expressed connexin in the ventricles and is normally located at the intercalated discs.6,7 Today, there are several lines of evidence that not only the total amount of Cx43 but also the distribution of this connexin is crucial for normal ventricular function and normal impulse propagation and it has been shown by several working groups that in various cardiac diseases with pathological left ventricular wall tension Cx43 expression and distribution is changed and that these connexin alterations may be 1 mechanism leading to life-threatening arrhythmias.8-11 On the other hand, one could assume that normal ventricular wall tension is essential for the formation of a regular Cx43 pattern and for the formation of a normal cardiomyocyte shape. Moreover, mechanical stretch as a physi-
ologial stimulus might be essential for cardiac development, and the arrangement of cardiomyocytes is essential for effective contractions. Thus, hypothetically a certain directed stretch applied on cardiomyocytes might promote Cx43 assembly at the intercalated discs and could also promote orientation of the cardiomyocytes. To test this hypothesis, we used a cell culture model of neonatal rat cardiomyocytes grown on flexible silicon membranes until confluence and subjected to controlled cyclic mechanical stretch (CMS) for 24 hours. We used this incubation time to assess changes in Cx43, because, Cx43 has a short half-life and an incubation period of 24 hours would allow a several-fold turnover of Cx43 proteins. Cx43 expression, distribution, and signal transduction pathways were analyzed. Because in many cardiac diseases, which go along with changed Cx43 localization, angiotensin II is increased we applied angiotensin II with and without CMS in additional experiments.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Cell Culture**

Cardiomyocytes were isolated and cultured as previously described. The cells were seeded at a density of 10^5 cells/cm² on gelatin-coated deformable silicone membranes (FlexCell cell culture plates, Dunn Labortechnik, Asbach, Germany) and were subjected to uniaxial, pulsatile stretch (105%, 110%, and 120% of resting length, 1 Hz) for 24 or 48 hours using the FlexCell Tension System FX-4040 (Dunn Labortechnik). Nonstretched cardiomyocytes seeded on FlexCell cell culture plates served as controls.

The percentage of nonmyocardial cells (fibroblasts, endothelial cells) was <5%, as revealed by specific immunocytochemistry (prolyl-4-hydroxylase; von Willebrand factor) and did not change during stretch.

After 24 or 48 hours, Cx43 expression was analyzed using immunocytochemistry, immunoblotting, or real-time PCR; moreover, phosphorylated proteins of extracellular signal-regulated kinase (ERK1/2) (also p42/44), glycogen synthase kinase (GSK3)β (Western blot), AKT (ELISA), and the transcription factors activator protein (AP)1 and cAMP response element–binding protein (CREB) (electrophoretic-mobility shift assay [EMSA]) were investigated as described previously and below.

**Immunocytochemistry**

To investigate whether CMS may affect the organization of the cellular communication with regard to the localization of the gap junctions, we processed the cells for troponin I- or α-actinin- and Cx43-immunocytochemistry and performed morphometric analysis in the following manner: as described above cells were exposed to 0, 24, or 48 hours of CMS with 5, 10 or 20% elongation. Additionally, in 1 series cells were allowed to recover from 24 hours cyclic stretch for another 24 hours at rest to test for reversibility of the changes. In a third series of experiments cells were stretched for 24 hours (10% elongation) and morphometric analysis of the cell adhesion molecule N-cadherin to Cx43 and N-cadherin expression as described previously. Briefly, the longitudinal cell axis was determined and divided into 4 sections of equal length giving 4 areas: the left and right cell pole and the 2 mid areas as depicted in Figure 1. We measured the number of cells with an elongated phenotype, which was defined as cells with a length/width ratio >2. In these cells, we also determined the angle between the longitudinal cell axis and the direction of stretch. Regarding the Cx43 and N-cadherin localization we determined polar and lateral Cx43 and N-cadherin expression as described previously.

**Western Blots**

Western blots of Cx43, of total and phosphorylated ERK1/2 and GSK3β, and GAPDH (as loading control) were carried out as previously described using standard protocols. Moreover, to test whether membrane proteins apart from gap junction channels may be influenced by stretch Na⁺/K⁺ ATPase and L-type Ca²⁺ channels were determined by Western blot (for details, see the Online Data Supplement).

**Reverse Transcription and PCR Amplification**

RNA isolation and reverse transcription was carried out using standard protocols. Real-time PCR was performed on the Light-

**Non-standard Abbreviations and Acronyms**

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<tr>
<th>Abbreviation</th>
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<tr>
<td>AKT</td>
<td>protein kinase B</td>
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<tr>
<td>AP1</td>
<td>activator protein 1</td>
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<tr>
<td>BIM I</td>
<td>bisindolylmaleimide I</td>
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<td>CMS</td>
<td>cyclic mechanical stretch</td>
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<td>Cx43</td>
<td>connexin43</td>
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<td>CREB</td>
<td>cAMP response element–binding protein</td>
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<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<td>EMSA</td>
<td>electrophoretic-mobility shift assay</td>
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<td>ERK</td>
<td>extracellular signal–regulated kinase</td>
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<td>GSK3β</td>
<td>glycogen synthase kinase 3β</td>
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<td>H8</td>
<td>N-(2-methlyamino-ethyl)-5-isouquinoline-sulphonamid</td>
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<tr>
<td>MEK1/2</td>
<td>mitogen-activated protein kinase 1/2 kinase</td>
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<td>PD98059</td>
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Figure 1. A, Schematic drawing elucidating the morphometric analysis of the cells. B–D, Original immunofluorescent localization of Cx43 and N-cadherin in unstretched and stretched cardiomyocytes. For details, see Methods. B, Nonorientated cardiomyocytes (static) and elongated cardiomyocytes (24-hour cyclic stretch, 10%, 1 Hz) stained for troponin I (red) and Cx43 (green). Nuclei are counterstained with DAPI (blue). Note the polarization of Cx43 (white arrows) in stretched cardiomyocytes. C, Nonorientated cardiomyocytes (static) and elongated cardiomyocytes (24-hour cyclic stretch, 10%, 1 Hz) stained for α-actinin (red) and Cx43 (green). Nuclei are counterstained with DAPI (blue). Note the polarization of Cx43 (white arrows) in stretched cardiomyocytes. D, Nonorientated cardiomyocytes (static) and elongated cardiomyocytes (24-hour cyclic stretch, 10%, 1 Hz) stained for N-cadherin (red) and Cx43 (green). Nuclei are counterstained with DAPI (blue). Note the parallel polarization of N-cadherin and Cx43 (white arrows) in stretched cardiomyocytes. B through D, The stretch axis is indicated by a yellow arrow.
Cycler 480 (Roche, Mannheim, Germany) using the following primer pairs:

- Cx43 antisense: 5’-H11032-GGTGGGCACAGACAGAATAT-3’/H11032
- Cx43 sense: 5’/H11032-CTCAACAACCTGGCTGCGAAA-3’/H11032
- GAPDH antisense: 5’/H11032-TCCTGGAAGATGGTGATGGGTTTC-3’/H11032
- GAPDH sense: 5’/H11032-ACATGTTCCAGTATGACTCTACCCAC-3’/H11032

At the end of each PCR, the relative amount of Cx43-mRNA in comparison to the mRNA of the housekeeping gene GAPDH was evaluated as previously published.14,18 For details, see the Online Data Supplement.

**Sandwich ELISA**

To evaluate the phosphorylated forms of AKT, a detection kit from New England Biolabs (Frankfurt, Germany) was used. Cell lysates were added to the microtiter plates coated with either the primary antibody against phosphorylated (Ser473) or total AKT, respectively, and incubated at 4°C overnight. Thereafter, the detection protocol was carried out according to the manufacturer’s instructions (for details, see the Online Data Supplement).

The phosphorylated forms of AKT were evaluated in relation to the total AKT (phosphorylated and nonphosphorylated forms), respectively, and the signals of the experimental (stretched) groups were compared to the signals of the untreated control cells (no stretch).

**Preparation of Nuclei for EMSA**

The preparation of nuclear extracts was carried out as described previously14 using the nuclear extraction kit from BioCat (Heidelberg, Germany) (see also the Online Data Supplement).

**Electrophoretic Mobility-Shift Assay**

To evaluate the transcription factors AP1 and CREB we also used a detection kit from BioCat. The shift assays were carried out as previously published14 and according to the instructions of the manufacturer. The specific bands were scanned, digitized, and analyzed as published (see also the Online Data Supplement).14

**Statistical Analysis**

For statistical analysis, ANOVA was performed, and if ANOVA indicated significant differences (P<0.05), the data were additionally analyzed with Student’s t test for paired or unpaired observations and corrected for multiple comparisons if necessary. For statistical analysis,
the software Systat for Windows, version 11 (Systat Inc, Evanston, Ill) was used. All data are given as means±SEM.

**Results**

**Immunocytochemistry**

We found that 24 hours CMS resulted in an increased percentage of cells with elongated phenotype. This was dependent on the intensity of stretch: 10% or 20% stretch generated significantly more elongated cells than 5% stretch. However, there was no difference between 10% and 20% stretch (Figure 2A). Moreover, the maximum number of elongated cells at 20% stretch was reached after 24 hours (Figure 2B).

Cells of the elongated phenotype were orientated 79±8° toward the stretch direction, ie, they were not parallel in the stretch axis, but transverse to it (original histological slides stained for troponin I and Cx43 are presented in Figure 1B; original slides stained for α-actinin and Cx43 are presented in Figure 1C).

Regarding localization of Cx43, we found in the elongated phenotype that Cx43 was preferentially accentuated at the cell pole in dependence on stretch intensity (Figure 3A) reaching maximum orientation after 24 hours of stretch exposure (Figure 3B). Moreover, staining of the cells (stretched and nonstretched) for both Cx43 and N-cadherin revealed that parallel to Cx43 also N-cadherin was accentuated at the polar membrane of elongated cells (nonstretched cells: N-cadherin ratio pole/middle: 1.45±0.13; stretched cells N-cadherin ratio pole/middle: 6.25±1.4; original histological slides stained for N-cadherin and Cx43 are presented in Figure 1D).

![Figure 4. Reversible and nonreversible effects of stretch and pharmacologic intervention. A, The first 3 columns show the nonreversibility of the effect of 24-hour (10%, 1 Hz) CMS within 24 hours of rest with regard to the percentage of elongated cells. Columns 4 and 5 show the effect of 0.1 μmol/L angiotensin on nonstretched and stretched cells (24 hours, 10%, 1 Hz) with regard to the percentage of elongated cells. B, The first 3 columns demonstrate the reversibility of the effect of 24-hour (10%, 1 Hz) CMS within 24 hours of rest with regard to the polar presence of Cx43. Columns 4 and 5 show the effect of 0.1 μmol/L angiotensin on nonstretched and stretched cells (24 hours, 10%, 1 Hz) with regard to the polar presence of Cx43. C, The first 5 columns indicate the effect of the MEK1 inhibitor PD98059 (10 μmol/L), the PKC inhibitor BIM I (6 μmol/L) and the PKA inhibitor H8 (2 μmol/L) on the percentage of elongated cells (24 hours cyclic stretch, 10%, 1 Hz). Columns 6 to 10 show the effect of these inhibitors on Cx43 polarization (24 hours cyclic stretch, 10%, 1 Hz). All values are given as means±SEM. *P<0.05, significant differences vs nonstretched control cells (indicated as 0); $P<0.05$, significant differences vs 0.1 μmol/L angiotensin (0+angiotensin); #P<0.05, significant differences vs 24 hours stretch.](http://circres.ahajournals.org/ытаhttp://circres.ahajournals.org/ły/fig/f4.png)
To test for reversibility of these changes, we submitted cells to 24 hours CMS (1 Hz, 10% elongation), followed by a period of 24 hours rest (no stretch) for recovery. It became evident that the percentage of cells with elongated phenotype was not reversible within this period (Figure 4A, columns 1 to 3), whereas, in contrast, the polar accentuation of Cx43 was clearly reversible and nearly reached the control level (ie, 0 hours of stretch) within 24 hours of rest after 24 hours of stretch (Figure 4B, columns 1 to 3).

Because it is well known that mitogen-activated protein kinase signaling pathways as well as angiotensin are involved in cardiac remodelling processes, we wanted to elucidate the effect of stretch with simultaneous blockade of MEK1 (upstream of ERK1/2) on one hand and the effect of stretch and concomitant application of angiotensin on the other hand. Angiotensin had no influence on the number of elongated cells and no influence on Cx43 distribution, but prevented from the stretch-induced Cx43 polarization (Figure 4A and 4B, columns 4 and 5).

Simultaneous treatment of cardiomyocytes with cyclic stretch (24 hours, 10%, 1 Hz) and the MEK1 inhibitor 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059) (10 μmol/L) revealed that PD98059 had no effect on cell orientation (Figure 4C, columns 1 to 3), but completely prevented from stretch-induced polarization of Cx43. The Cx43 ratio pole/middle was significantly reduced from 6.72±1.10 (stretch alone) to 1.55±0.2 (stretch+PD98059) and thus reached control levels (Figure 4C, columns 6 to 8). Moreover, to evaluate whether other pathways might play a role in the stretch dependent changes in cell morphology and Cx43 distribution the protein kinases A and C were examined: inhibition of these 2 kinases did not change neither the percentage of elongated and orientated cells nor the ratio Cx43 pole/middle (Figure 4C, columns 4 and 5 and 9 and 10).

**Western Blot, ELISA, PCR, and EMSA Results**

Application of cyclic stretch for 24 hours resulted in a significant 40% upregulation of Cx43 protein expression whereas GAPDH expression remained unaltered by this treatment (Figure 4A, columns 1 and 2). The ratio P-Cx43/NP-Cx43 was not significantly altered by stretch (non-stretched cells: P-Cx43/NP-Cx43: 2.89±0.66; stretched cells: 2.94±0.66).
P-Cx43/NP-Cx43: 3.16±0.93). The enhanced Cx43 protein expression was accompanied by a significant 38% increase in Cx43 mRNA as revealed by real-time PCR (Figure 5A, columns 4 and 5). Again the house keeping gene GAPDH remained unaltered by cyclic stretch. Moreover, the stretch-induced upregulation of Cx43 protein was reversible within 24 hours after end of cyclic stretch (Figure 5B, columns 1 to 4). Because Polontchouk et al demonstrated that angiotensin II increases total Cx43 protein level we also tested angiotensin II (0.1 μmol/L) in our system: application of both stimuli (stretch+angiotensin) revealed that Cx43 protein amount increased but not in an additive way. Hence, the Cx43 signal with stretch and angiotensin did not exceed the signal with stretch alone (Figure 5B, column 5).

For further investigation of the underlying signaling pathways we examined the influence of possibly relevant kinases on the stretch induced Cx43 upregulation. Because it is known that ERK, AKT and GSK3β are involved in the signal transduction of stretch19,20 Western blot analysis was carried out on cardiomyocytes exposed to cyclic stretch for 24 hours and the phosphorylated forms of ERK1/2 and GSK3β were investigated. After stretch application we found a significant upregulation of the phosphorylated forms of ERK1/2 (P-ERK1/2) and GSK3β (P-GSK3β) (Figure 6A, columns 1 to 6). Moreover, because it is known that AKT phosphorylates and thereby inactivates GSK3β21 the phosphorylated isoform of AKT was also determined by ELISA. These experiments showed that after cyclic stretch AKT becomes significantly phosphorylated and thereby activated (Figure 6A, columns 7 and 8). The P-ERK1/2 result was confirmed in experiments in which the selective inhibitor of the MEK1 PD98059 was administered concomitantly to stretch application to inhibit ERK1/2 phosphorylation. In these experiments the stretch-dependent upregulation of Cx43 protein and mRNA was...
Furthermore, because it is known that binding sites for AP1 and CREB exist within the Cx43 promoter, the influence of stretch on the nuclear content of AP1 and CREB was examined using the EMSA technique. Application of cyclic stretch (10%, 24 hours) resulted in a significant upregulation of both transcription factors in the nucleus (Figure 7A and 7B), as assessed by the positive shift of these transcription factors in the nuclear fractions of stretched cells.

Discussion

The immunohistological findings showed elongation and Cx43 polarization within 24 hours of CMS. Interestingly, the orientation of the longitudinal cell axis was not in direction of stretch but transverse to it. One may imagine that this resembles a feature of self-organization of the myocardium. The orientation transverse to the stretch axis allows the cells to absorb the transforming force by their contraction. Orientation parallel to the stretch axis would result in large forces bending the cells apart and would probably overload the side-to-side cell adhesion mechanisms. Thus, the transverse orientation probably is more stable by allowing the cells to counteract the distracting forces by the cells contraction. The polarization of Cx43, in addition, may allow the action potential to spread in the longitudinal direction, so that the resulting contraction (ca. 70 to 90° toward the stretch axis) can be coordinated between the cells (see schematic diagram, Figure 8). Moreover, in our model of directed cellular stretch the Cx43 alignment at the cell poles was reversible after a rest period of 24 hours in contrast to the elongation of the cardiomyocytes, which was irreversible within this time period. The reversibility of the Cx43 polarization is in good accordance with the high turnover of Cx43 in the membrane with half-life times of 1 to 3 hours and may indicate that cardiomyocytes can adapt the localization of Cx43 to changing mechanical situations.

Our findings that cardiomyocytes adjust their morphology in such a way that they elongate and rearrange to stretch direction with an angle of ≈80° are in good accordance with the results of Matsuda et al. and de Jonge et al. These authors demonstrated that cardiomyocytes exposed to cyclic stretch show cell elongation and a rearrangement of F-actin fibers. Interestingly, Matsuda et al. also found an orientation of ≈50% of the cardiomyocytes after 24 hours of stretch with an angle between 60° and 90° of the long cell axis in relation to stretch direction. The polarization of Cx43, however, has not been investigated in these studies, and seems to be related to the process of orientation of the fibers. Besides the Cx43 polarization we found that N-cadherin (known to be closely apposed to Cx43) also polarizes in dependence on stretch, which is in good accordance to Matsuda et al. who also found a colocalization of Cx43 and N-cadherin at the longitudinal cell termini. However, although cell orientation was independent from ERK1/2, Cx43 polarization seemed to depend on ERK1/2 as this could be inhibited by PD98059 (see results and below). Thus, these data suggest that cell orientation and elongation might involve other processes than Cx43 polarization.

completely abolished when ERK1/2 phosphorylation was blocked (Figure 5A, column 6; Figure 6B, columns 1 to 2 and 5 to 6). The ratio of phosphorylated Cx43 versus nonphosphorylated Cx43 was not significantly affected by PD98059 inhibition of ERK1/2 (nonstretched cells +PD98059: P-Cx43/NP-Cx43: 2.51±0.51; stretched cells +PD98059: P-Cx43/NP-Cx43: 2.87±0.91), whereas the polar accentuation of Cx43 induced by stretch (see results above) was abolished by simultaneous PD98059 treatment and reached the control level.

In contrast, inhibition of the PKC or PKA did not significantly alter the stretch-induced upregulation of Cx43 protein (Figure 6B, columns 3 to 4 and 7 to 8) neither the polar accentuation of Cx43 (Figure 4C, columns 4 to 5 and 9 to 10).

To test whether other membrane proteins might be influenced by stretch, we also examined the Na⁺/K⁺-ATPase and the L-type Ca²⁺ channel: both proteins did not show significant alterations in their expression level after 24 hours of stretch (Figure 6C and 6D).

Figure 7. Stretch-induced translocation of transcription factors. A, Upregulation of the transcription factors AP1 and CREB after 24 hours of stretch application (10% elongation, 1 Hz) measured by EMSA. *P<0.05, significance vs control (nonstretched cells). All data are given as means±SEM of n=6 experiments. B, Original AP1 and CREB EMSA from nonstretched control cells and from cells after 24 hours (10% elongation, 1 Hz) of stretch. Note the positive AP1 and CREB shift, respectively, in both nuclear extract preparations (2), which was more pronounced after stretch application and the negative AP1 or CREB shift in the absence of cell extracts (1). con indicates nonstretched control. The cytosolic preparations (3) showed no change whatsoever after stretch application.

Figure 8. Schematic diagram illustrating the effect of cyclic stretch on the myocardium. The direction of the stretch is indicated by the bar. The myocardial chamber can be divided into longitudinal (parallel to the stretch axis) and transverse sections (transverse to the stretch axis). The myocardial fibers (F) run in the longitudinal direction and form a network of cross bridges between the sarcomeres, which are responsible for the contraction of the myocardium (cell bundle). The location of the transverse sections (TS) is indicated by the arrows. The myocardial fibers can be oriented either parallel to the stretch axis or transverse to it. One may imagine that this resembles a feature of self-organization of the myocardium. The cardiomyocytes can adapt the localization of Cx43 to changing mechanical situations.
Besides the altered morphology of the cardiomyocytes, we also found not only Cx43 polarization but also an increase in total Cx43 protein and mRNA after stretch application, whereas Na⁺/K⁺-ATPase and L-type Ca²⁺-channel expression were not altered. These findings are in line with results presented by Wang et al., Shyu et al. and Shanker et al. These authors demonstrated that Cx43 is upregulated following stretch, however, without investigating the intracellular localization of Cx43.

Another point to address is the response of Cx43 protein to the combination of stretch and angiotensin II. Shyu et al demonstrated that angiotensin was released by stretch and stimulates Cx43 synthesis dependent on the angiotensin-receptor 1. In our experiments, we found that concomitant application of stretch and angiotensin did increase Cx43 protein levels, but the increase was in the same order of magnitude as a sole stretch application. Thus, it might be imaginable that a ceiling effect of a yet unknown mechanism prevents from further increases of the Cx43 signal.

To unravel the underlying signal transduction pathway and because it is known that ERK, AKT and GSK3β are involved in the signal transduction of stretch, we examined the phosphorylated forms of ERK1/2 and GSK3β by Western blot and of AKT by ELISA. Our results indicated that all these 3 signal transduction proteins are phosphorylated after 24 hours of stretch and, thus, are in good accordance with literature. This means that stretch activates ERK1/2 (phosphorylated form is active) and inactivates GSK3β (phosphorylated form is the inactive form). Because our previous studies on statically grown cardiomyocytes revealed a dependence of Cx43 expression on ERK1/2 activation, it might be imaginable that induction of the mitogen-activated protein kinase signaling pathway by stretch contributes to the enhancement of Cx43. Thus, we evaluated the role of ERK1/2 by inhibiting its key phosphorylation enzyme MEK1 with PD98059 and could demonstrate that the stretch-induced Cx43 upregulation and polarization was completely abolished by PD98059 treatment.

On the other hand, our data with the PKC inhibitor BIM I and the PKA inhibitor H8 revealed that obviously these 2 kinases are not involved in the stretched induced Cx43 upregulation, which is in good accordance to de Jonge et al., who demonstrated that endothelin-1 but not CMS activated the PLCβ-PKC cascade.

Furthermore, it is known that binding sites for AP1 (which is downstream of ERK1/2) and CREB exist within the Cx43 promoter. Therefore, we wanted to examine if stretch induces nuclear accumulation of these transcription factors. In our EMSA experiments enhanced localization of both factors in the nuclei of stretched cells could clearly be demonstrated and, because previous studies of our working group indicated a dependence of Cx43 expression on nuclear presence of AP1 and CREB after ERK1/2 activation by isoprenaline, it might be possible that these transcription factors are upregulated following ERK1/2 activation by stretch. There are reports supporting this hypothesis showing an elevation of AP1 as well as CREB after ERK1/2 phosphorylation. Because it is known that mechanical stretch induces ERK1/2 activation it might be conceivable that stretch activates mitogen-activated protein kinase signaling pathways and, thus, results in Cx43 upregulation and polarization.

Interestingly, in contrast to the Cx43-response regarding protein expression, the stretch-induced orientation and elongation of the cells seems to be independent from ERK1/2, because this was not affected by PD98059, indicating that additional other signal cascades also seem to be activated by stretch. Moreover, PKC and PKA do not seem to be involved.
in the stretch-response investigated here, because BIM I and H8 both did not affect cell orientation or Cx43 polarization or expression. Our finding that angiotensin II inhibited the polar accentuation of Cx43 is in good agreement with clinical findings that in cardiac diseases associated with elevated angiotensin and enhanced wall stretch such as dilative cardiomyopathy Cx43 was found to be reduced at the cell poles and was enhanced at the lateral sides of the cells.8–11 This points to a role for angiotensin II in dysregulation of Cx43 localization under stretch.

Thus, our data show a self-organization of cardiomyocytes under CMS with orientation transverse to the stretch axis and polarization of Cx43, which may allow a counteraction of the distracting forces of stretch by contraction and may help to organize a coordinated wave-like contraction of the tissue by anisotropic distribution of Cx43. Along with this process, ERK1/2 and AKT are activated. As a result, Cx43 is upregulated on the one hand; and, on the other hand, GSK3-β is phosphorylated and thereby inactivated, which might induce hypertrophic processes.

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Disclosures
None.

References

1055–1063.


What Is Known?

- Gap junction channels form the basis of intercellular communication and play an important role for cellular growth and differentiation.
- Cardiomyocytes can elongate and orientate in response to mechanical stretch, but it is unclear whether this process also involves the intracellular orientation of gap junctions.
- Expression of the gap junction protein connexin (Cx)43 at the lateral sides of myocytes is a common feature in many cardiac diseases (heart failure, atrial fibrillation, chronic infarction) associated with enhanced stretch and load, but it is unknown how this process might be regulated.

What New Information Does This Article Contribute?

- Within 24 hours of exposure to CMS, cultured cardiomyocytes exhibit elongation and orientation transverse (79°) to the stretch axis as well as increased levels of Cx43 with accentuated localization of Cx43 at the cell poles, which is reversible within 24 hours at static conditions, whereas elongation and orientation are not reversible.
- Angiotensin II does not affect stretch-induced elongation or orientation of the cells but inhibits the polarization of Cx43 leading to lateralization of Cx43.
- The stretch-induced expression of Cx43 and its polar localization are transduced via ERK1/2 (extracellular signal-regulated kinase 1/2) involving the transcription factors AP1 (activator protein 1) and CREB (cAMP response element–binding protein), whereas cellular elongation and orientation are independent of ERK1/2.

Although much is known about cardiac pathophysiology, the processes by which mechanical stretch affects growth, differentiation, or organization of cardiomyocytes are poorly understood. However, this is of special interest, because cyclic stretch and changes in mechanical stretch typically accompany many cardiac diseases or occur during cardiac development. We examined whether CMS or a combination of stretch and angiotensin II can affect the localization of gap junctions with regard to the cell axis. Cyclic mechanical stretch leads to self-organization of cardiomyocytes with (nonreversible) orientation transverse to the stretch axis and (reversible) polarization of Cx43, which may allow a counteraction of distracting forces of stretch by contraction and may help to organize a coordinated wave-like contraction of the tissue by anisotropic distribution of Cx43. Along with this process, ERK1/2 and AKT are activated and as a result Cx43 is upregulated and accentuated at the cell poles. Cardiomyocyte orientation and elongation, however, are independent from ERK1/2 activation. Angiotensin II inhibits the polar accentuation of CX43 but does not affect elongation or orientation of the cell axis. This indicates that cardiomyocytes can adapt the localization of Cx43 to changing mechanical situations, which might lead to altered pathways of electric propagation.
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Methods:

Cell culture:
Cardiomyocytes were isolated and cultured as previously described1. Briefly, ventricles of new-born Wistar rats were digested in collagenase solution, centrifuged, and, after a preplating period to remove non-myocardial cells, resuspended in M199 medium (Invitrogen, Karlsruhe, Germany) containing 2 mmol/L L-glutamine, 100 mg/L streptomycin and penicillin, 1% foetal calf serum, and 10% horse serum (to inhibit fibroblast growth). The cells were seeded at a density of $10^5$ cells/cm$^2$ on gelatine coated deformable silicone membranes (FlexCell cell culture plates, Dunn Labortechnik, Asbach, Germany) and were subjected to uniaxial, pulsatile stretch (105%, 110% and 120% of resting length, 1Hz) for 24 or 48 hours using the FlexCell Tension System FX-4000 (Dunn Labortechnik, Asbach, Germany). Non-stretched cardiomyocytes seeded on FlexCell cell culture plates served as controls. Moreover in some experiments angiotensin-II (0.1µmol/L, Alexis, Loerrach, Germany) or the MEK 1/2 inhibitor PD98959 (10µmol/L, Alexis, Loerrach, Germany) or the PKA inhibitor H8 (2µmol/L, Alexis, Loerrach, Germany) or the PK C inhibitor BIM I (5µmol/L, Alexis, Loerrach, Germany) were administered simultaneously to stretch application. The percentage of non-myocardial cells (fibroblasts, endothelial cells) was <5% as revealed by specific immunocytochemistry (prolyl-4-hydroxylase; von Willebrand´s factor) and did not change during stretch. After 24 or 48 hours Cx43 expression was analysed using immunocytochemistry, immunoblotting or real-time PCR, moreover, phosphorylated-proteins of ERK, GSK3β (Western Blot), AKT (ELISA) and the transcription factors AP1 and CREB (EMSA) were investigated as described previously1,2 and below.

Western blots:
Briefly, after 24 hours treatment, cells were harvested and lysed at 4°C applying three strikes of ultrasound for 10s each using a low low-salt buffer with inhibitors of proteases and phosphatases (10 µg/mL aprotinin, 10 µg/mL leupeptin, 10 µg/mL pepstatin A, 10 nmol/L okadaic acid, 100 µmol/L phenylarsinoxide, 100 µmol/L cantharidin, 0.1 mmol/L sodiumorthovanadate, 10 mM sodiumpyrophosphate, 20 mM Na$_3$PO$_4$, 150 mM NaCl, 2 mM MgCl$_2$, 0.1 % Nonidet P40, 1% Triton X-100, 1% SDS, 10% glycerol). Total protein concentration was determined using standard protocols. Thereafter, whole cell lysates were mixed with gel-loading buffer, according to Laemmli following classical protocols and for electrophoresis 20 µg of protein per slot was fractionated through a 4% stacking and a 10%
running SDS-polyacrylamide gel. Proteins were then transferred on to a PVDF membrane using the wet-blot technique and blocked with 5% low-fat milk at 4°C overnight. Primary antibodies (as described below; and see: “Materials”) were applied for 2 hours at room temperature and the following dilutions were used: for Cx43 1:5000, for GAPDH 1:10000, for Na⁺/K⁺-ATPase 1:1000, for L-type Ca²⁺-channel 1:1000, for phosphorylated ERK1/2 1:1000, for total ERK1/2 1:1000, for phosphorylated GSK3β 1:1000 and for total GSK3β 1:2000. Thereafter, the blots were washed with TRIS-buffered saline (TBS) containing: 500 mmol/L NaCl, 50 mmol/L TRIS-HCL (pH 7.4) and 0.1% Tween 20 and were incubated with the appropriate secondary horseradish peroxidase-labelled antibody (dilutions: 1:5000 for Cx43 and GAPDH; 1:1000 for phosphorylated ERK1/2 and 1:2000 for ERK1/2, total GSK3β and phosphorylated GSK3β) for 1 hour at room temperature. Subsequently, the detection was carried out using the iodophenol/luminol system by application of ECL (enhanced chemiluminescence) Western blot detection kit from Thermo Fisher Scientific (Dreieich, Germany). The blots were incubated according to the manufacturer’s instructions for 60s with the reaction mixture and then exposed to X-ray film to detect chemiluminescence. The specific bands were imaged on a scanner, digitised and analysed with BioRad software (BioRad, München, Germany). After background subtraction gray scale values of the specific signals in the stretch group were compared with signals of the non-stretched control cells. All bands were normalised to GAPDH content (assessed after stripping and reprobing of the blots by the same method as described above). The phosphorylated forms of ERK1/2 and GSK3β were evaluated in relation to total ERK 1/2 and GSK3β proteins (i.e. phosphorylated and non-phosphorylated-forms) respectively, and again the signals of the stretch group were compared to the signals of the non-stretched control cells.

Reverse transcription and PCR amplification:
RNA was isolated using Trizol (Gibco BRL, Karlsruhe, Germany). Thereafter, RNA was reverse transcribed from 1 μg total RNA with random hexamers to generate first-strand cDNA using the DyNAmo cDNA synthesis kit from New England Biolabs (Frankfurt, Germany). After first-strand cDNA was prepared, 1μl cDNA was mixed with PCR reagents using DyNAmo Flash Sybr Green qPCR kit from New England Biolabs (Frankfurt, Germany) according to the manufacturer’s instruction to make a 25μL solution and real-time PCR was carried out on the Light-Cycler 480 (Roche, Mannheim, Germany) using the following primer pairs:

Cx43  
antisense 5'-GGTGGGCACAGACACGAATAT-3'
sense 5'-CTCAACAACCTGGCTGCGAAA-3'

GAPDH  
antisense 5'-TCCTGGGAAGATGGGTGATGGGTTC-3'
sense 5'-ACATGTTCCAGTGACCTACTCCAC-3'
At the end of each PCR-run the relative amount of Cx43-mRNA in comparison to the mRNA of the housekeeping gene GAPDH was evaluated as previously published\(^1\), according to Livak and Schmittgen (2001)\(^3\), using the 2\(^{-\Delta\Delta Ct}\)-method. Briefly, using this method the data are presented as the fold in gene expression normalised to the housekeeping gene GAPDH and relative to the untreated control. From the Ct-values (defined as the threshold cycle at which the SybrGreen fluorescence exceeds background fluorescence as automatically determined by the light-cycler), for both genes the \(\Delta Ct\)-values were calculated as Ct\(_{Cx43}\)-Ct\(_{GAPDH}\). The data were then analysed according to the following equation: 
\[
\Delta\Delta Ct = \Delta(Ct_{Cx43}-Ct_{GAPDH})_{\text{stretch}} - \Delta(Ct_{Cx43}-Ct_{GAPDH})_{\text{no stretch}}.
\]
From the \(\Delta\Delta Ct\) values the term 2\(^{-\Delta\Delta Ct}\) was calculated.

**Sandwich-ELISA:**
To evaluate the phosphorylated forms of AKT a detection kit from New England Biolabs (Frankfurt, Germany) was used. Cells were harvested and lysed using the following lysis buffer: 20 mmol/L TRIS (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodiumpyrophosphate, 1 mmol/L β-glycerolphosphate, 1 mmol/L sodiumorthovanadate, 1 μg/mL leupeptin and 1 mmol/L PMSF.

The cell lysates were added to the microtiter plates coated with either the primary antibody against phosphorylated-(Ser473) or total-AKT respectively and incubated at 4°C over night. Thereafter, according to the manufacturer’s instructions the cells were incubated with a secondary detection antibody for 2 hours at room temperature and after several washing steps with the third horseradish peroxidise-labelled antibody. The dye reaction was carried out using TMB (3,3’,5,5’-tetramethylbenzidine) and dye development was evaluated at 450nm using an ELISA-reader (Tecan, Groedig, Austria). The phosphorylated-forms of AKT were evaluated in relation to the total-AKT (phosphorylated and non-phosphorylated-forms), respectively and the signals of the experimental (=stretched) groups were compared to the signals of the untreated control cells (no stretch).

**Preparation of nuclei for EMSA (electromobility-shift-assay):**
The preparation of nuclear extracts was carried out using the nuclear extraction kit from BioCat (Heidelberg, Germany). The cells were harvested, according to the manufacturer’s protocol with a buffer containing 10 mmol/L HEPES pH 7.9, 10mmol/L KCl, 10 mmol/L EDTA, 10 μL 100 mmol/L DTT, 10 μL protease inhibitor-cocktail and 40 μL 10% Igepal (tert-octylphenoxy poly(oxyethylene)ethanol). The cells were centrifuged (3 min, 15000g) and the pellets containing the nuclear fraction were resuspended in the following buffer: 20 mmol/L HEPES pH 7.9. 100 mmol/L NaCl, 1 mmol/L EDTA, 10% glycerol, 1.5 μL 100 mmol/L DTT and 1.5 μL protease inhibitor-cocktail and were vigorously agitated for 2 hours at 4°C. After a
second centrifugation step (5 min, 15000 g) the supernatants, now containing the nuclear extracts, were harvested and protein concentration was determined. Furthermore, to test the purity of the extracts the LDH-content (an enzyme found only in the cytoplasm of cells) was measured in the nuclear and cytosolic fractions using the "Cytotoxicity Detection KitPlus" from Roche Applied Science (Mannheim, Germany). Only nuclear fractions with less than 5% contamination with cytoplasm were used for the subsequent EMSA experiments.

**EMSA:**
To evaluate the transcription factors AP1 and CREB we also used a detection kit from BioCat (Heidelberg, Germany). According to the manufacturer's protocol 5µg of nuclear extract was mixed with the reaction buffer and the biotinylated-DNA-consensus sequence of AP1 (5’-CGCTTGATGACTCAGCCGGAA-3’) or of CREB (5’-AGAGATTTCCTGAGCTAGAGGCTAG-3’) (poly d(I-C) was added to block non-specific binding). After 30 min incubation at room temperature the probes were fractionated through a non-denaturating 4% polyacrylamide gel and transferred on to a nylon membrane using the wet-blot technique. Detection of the specific bands was carried out using horseradish peroxidase-labelled streptavidin and the iodophenol/luminol system. The blots were exposed to the reaction mixture for 5 min and then exposed to X-ray film to detect chemiluminescence. The specific bands were scanned, digitised and analysed as described above.

**Materials:**
The polyclonal antibodies (raised in rabbit) against the phosphorylated and non-phosphorylated forms of ERK1/2 and against the L-type Ca\(^{2+}\)-channel were purchased from Santa Cruz (Heidelberg, Germany) and the polyclonal antibodies (raised in rabbit) against the phosphorylated and non-phosphorylated forms of GSK3β were purchased from New England Biolabs (Frankfurt, Germany). Polyclonal Cx43 antibody raised in rabbit was obtained from Sigma-Aldrich (Steinheim, Germany), polyclonal Na\(^+\)/K\(^-\)-ATPase antibody raised in rabbit was obtained from Upstate (now Millipore, Schwalbach, Germany) and monoclonal GAPDH antibody raised in mouse from Acris (Hiddenhausen, Germany). The primers for Cx43 and GAPDH were bought from TIB MOLBIOL (Berlin, Germany). The cell culture media were purchased from Invitrogen (Karlsruhe, Germany). All other chemicals and secondary goat anti-rabbit or rabbit anti-mouse HRP-labelled antibodies were obtained from Sigma-Aldrich.
References:

