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Opposing and synergistic effects of cyclic mechanical stretch and α- or β-adrenergic stimulation on the cardiac gap junction protein Cx43

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ABSTRACT

In the heart the most prominent cardiac gap junction protein is connexin43 (Cx43). Increased Cx43 expression has been identified in cardiac hypertrophy and may contribute to arrhythmias. Besides acute effects on gap junction channel function, chronic regulation of Cx43 expression can affect intercellular communication. Since both cyclic mechanical stretch (CMS) and catecholamines play an important role in cardiac physiology and pathophysiology, we wanted to elucidate whether a prolonged β- or α-adrenoceptor stimulation may modulate the effects of CMS on Cx43 expression.

Neonatal rat cardiomyocytes were cultured on flexible 6-well plates. Thereafter, cells were kept static without any treatment or stimulated with 0.1 μmol/L isoprenaline or phenylephrine for 24 h without or with additional CMS (1 Hz; 10% elongation). Isoprenaline and phenylephrine given alone significantly increased Cx43-protein and -mRNA level. Also CMS resulted in a significant Cx43-protein and -mRNA up-regulation. The combined treatment of the cells with either isoprenaline or phenylephrine and stretch also resulted in an up-regulation of Cx43-protein and -mRNA, which did not exceed those of stretch, isoprenaline or phenylephrine alone. However, while CMS reduced the Cx43-protein/mRNA ratio, adrenergic stimulation increased Cx43-protein/mRNA ratio. While isoprenaline and phenylephrine increased Cx43-phosphorylation, additional CMS significantly reduced P-Cx43/Cx43 ratio.

For further investigation of the underlying signal transduction pathway, we examined the phosphorylated forms of ERK1/2, GSK3β and AKT and could demonstrate that these protein kinases are also significantly up-regulated following stretch or adrenoceptor stimulation. Again the combined treatment of cardiomyocytes with CMS and isoprenaline or phenylephrine had no additive effects.

Thus, the combination of α- or β-adrenoceptor stimulation and CMS up-regulates Cx43 expression and leads to phosphorylation of ERK1/2 and AKT (=activation) and of GSK3β (=inactivation). There were no significant additive effects compared to CMS or adrenergic stimulation alone indicating a possible ceiling effect. However, CMS and adrenergic stimulation differentially affected Cx43-protein/mRNA ratio and Cx43-phosphorylation.

1. Introduction

Intercellular communication is an important feature of organization within many types of tissue. Gap junction channels form the basis for direct intercellular communication. These channels also allow electrical and metabolic coupling between neighbouring cells and are important for a regular function of all organs. One complete gap junction channel is composed of two hemichannels of each neighbouring cell and the hemichannels (=connexons) themselves are composed of six protein subunits, the connexins [1]. Within the cell membrane a connexin is arranged as follows: it consists of four transmembrane domains, two extracellular and one intracellular loop. The N- and C-terminus are located at the cytoplasmatic side of the cell and it is known that the C-terminus contains various phosphorylation sites for a number of protein kinases, thereby regulating connexin assembly and disassembly [2–5]. In mammalian heart it has been shown that Cx43 is the predominantly expressed connexin in the ventricles, whereas Cx40 is the dominant connexin in the atria and in the conduction system. Cx45 is found in embryonic stages of the heart and in small

Abbreviations: AKT, protein kinase B; API, activator protein 1; BIM I, bisindolylmaleimide I; CMS, cyclic mechanical stretch; Cx43, connexin43; CREB, CRE-binding protein; Ct, threshold cycle; EMSA, electrophoretic mobility shift assay; ERK1/2, extracellular signal-regulated kinase 1/2 (=p42/44); GSK3β, glycogen synthase kinase 3b; GRK-2, G protein-coupled receptor kinase-2; PCR, polymerase chain reaction; PKC, protein kinase C.

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amounts also in the conduction system [6]. Cx43 exhibits a half-life time which is considerably short (90 min, [7]) as compared to other integral membrane proteins. It is normally found at the intercalated disks of cardiomyocytes which is a prerequisite for a normal stimulus conduction.

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, which has been discussed to be involved in the generation of an arrhythmogenic substrate leading to life-threatening arrhythmias [8–11].

Furthermore, in many cardiac diseases as dilated or hypertrophic cardiomyopathy catecholamines play an important role. It was shown by others that β- and α-adrenoceptors might participate in hypertrophic responses [12–13] and by our working group that β- or α-adrenoceptor stimulation alters Cx43 expression [14,15].

Moreover, we and others have recently shown that cyclic mechanical stretch can induce enhanced synthesis of Cx43 in cardiomyocytes [16–18]). However, an un-answered question still is, whether enhanced sympathoadrenergic stimulation may modulate the stretch response. This is important since in most situations of enhanced mechanical stretch the sympathoadrenal axis is activated. Thus, the aim of the present study was to clarify whether the stretch-induced up-regulation of Cx43 may be altered by submission of cultured cardiomyocytes to a combined treatment of cardiomyocytes with cyclic mechanical stretch and α- or β-adrenoceptor stimulation.

Therefore, we used a cell culture model of neonatal rat cardiomyocytes grown on flexible silicon membranes until confluence and subjected them to controlled cyclic mechanical stretch (CMS) for 24 h with additional stimulation of α- or β-adrenoceptors. We used this incubation time to assess changes in Cx43, because, as mentioned above, Cx43 has a short half-life and an incubation period of 24 h would allow a several-fold turnover of Cx43 proteins. Cx43 expression and signal transduction pathways were analysed using Western blot, ELISA, EMSA and real-time PCR techniques.

2. Methods

2.1. Cell culture

Cardiomyocytes were isolated and cultured as previously described [14]. 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 (110% of resting length, 1 Hz) without or with simultaneous administration of either the $\beta_1\beta_2$-adrenoceptor agonist isoprenaline (0.1 μmol/L, Sigma–Aldrich, Steinheim, Germany) or the α_1-adrenoceptor agonist phenylephrine (0.1 μmol/L, Sigma–Aldrich, Steinheim, Germany) or the protein kinase C (PKC) inhibitor bisindolylmaleimide I (5 μmol/L BIM I, Alexis Biochemicals, Loerrach, Germany) for 24 h using the FlexCell Tension System FX-4000 (Dunn Labortechnik, Asbach, Germany). Non-stretched cardiomyocytes seeded on FlexCell cell culture plates served as controls. Moreover, as a control and to corroborate our previously published data on the effect of isoprenaline and phenylephrine on Cx43 expression, in some experiments the $\beta_1\beta_2$-adrenoceptor agonist isoprenaline (0.1 μmol/L, Sigma–Aldrich, Steinheim, Germany) or the α_1-adrenoceptor agonist phenylephrine (0.1 μmol/L, Sigma–Aldrich, Steinheim, Germany) were administered without concomitant stretch application [14,15].

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 or isoprenaline or phenylephrine stimulation.

After 24 h Cx43 expression was analysed using immunoblotting and real-time PCR, moreover, phosphorylated-proteins of Cx43, ERK1/2, GSK3β (Western blot), AKT (ELISA) and the transcription factors AP1 and CREB (EMSA) were investigated as described previously [19] and below.

2.2. Western blots

Briefly, after 24 h treatment, cells were harvested and lysed at 4 °C applying three strikes of ultrasound for 10 s each using a 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_3PO_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 [20]. 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) were applied for 2 h at room temperature and the following dilutions were used: for Cx43 1:5000, for GAPDH 1:10000, 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 h 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 60 s 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 ('Quantity One' BioRad, München, Germany). After background subtraction gray scale values of the specific signals in the experimental groups were compared with signals of the non-stretched control cells. All bands were normalized 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 ERK1/2 and GSK3β proteins (i.e. phosphorylated and non-phosphorylated forms) respectively, and again the signals of the experimental groups were compared to the signals of the non-stretched control cells.

2.3. 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, 1 μL 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 25 μL solution and real-time PCR was carried out on the Light-Cycler 480 (Roche, Mannheim, Germany) using the following primer pairs: