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RESEARCH PAPER

The signal transduction cascade regulating the expression of the gap junction protein connexin43 by β-adrenoceptors

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Background and purpose: In mammalian heart, connexin43 (Cx43) represents the predominant connexin in the working myocardium. As the β-adrenoceptor is involved in many cardiac diseases, we wanted to clarify the pathway by which β-adrenoceptor stimulation may control Cx43 expression.
Experimental approach: Cultured neonatal rat cardiomyocytes were stimulated with isoprenaline. Cx43 expression as well as activation of p38 mitogen-activated protein kinase (MAPK), p42/44 MAPK, JUN NH_2-terminal kinase (JNK) and nuclear translocation of the transcription factors activator protein 1 (AP1) and CRE-binding protein (CREB) were investigated. Additionally, we assessed Cx43 expression and distribution in left ventricular biopsies from patients without any significant heart disease, and from patients with either congestive heart failure [dilated cardiomyopathy (DCM)] or hypertrophic cardiomyopathy (HCM).
Key results: Isoprenaline exposure caused about twofold up-regulation of Cx43 protein with a pEC_{50} of 7.92 ± 0.11, which was inhibited by propranolol, SB203580 (4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)-1H-imidazole) (p38 inhibitor), PD98059 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one) (MAPK 1 kinase inhibitor) (Alexis Biochemicals, San Diego, CA, USA) or cyclosporin A. Similar findings were obtained for Cx43 mRNA. Furthermore, Cx43 up-regulation was accompanied by phosphorylation of p38, p42/44 and JNK, and by translocation of AP1 and CREB to the nucleus. Analysis of Cx43 protein and mRNA in ventricular biopsies revealed that in patients with DCM, Cx43 content was significantly lower, and in patients with HCM, Cx43 content was significantly higher, relative to patients without any cardiomyopathy. More importantly, Cx43 distribution also changed with more Cx43 being localized at the lateral border of the cardiomyocytes.
Conclusion and implication: β-adrenoceptor stimulation up-regulated cardiac Cx43 expression via a protein kinase A and MAPK-regulated pathway, possibly involving AP1 and CREB. Cardiomyopathy altered Cx43 expression and distribution.
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Keywords: Cx43; cardiomyocytes; beta-adrenoceptor; isoprenaline; MAPK; congestive heart failure; hypertrophic cardiomyopathy; human cardiac biopsy

Abbreviations: AP1, activator protein 1; CREB, CRE-binding protein; Cx43, connexin43; H8, (N-[2-(methylamino)ethyl]-5-isoquinolinesulphonamide); JNK, JUN NH_2-terminal kinase; NFATc3, nuclear factor of activated T-cells; p38 MAPK, p38 mitogen-activated protein kinase; p42/44 MAPK, p42/44 mitogen-activated protein kinase; PD98059, (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one); PKA, protein kinase A; SB203580, (4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)-1H-imidazole)

Introduction

Intercellular communication is an important feature of organization within many kinds of tissue. Gap junction channels form the basis of direct intercellular communication. These

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channels allow electrical and metabolic coupling between neighbouring cells. One complete gap junction channel is composed of two hemichannels (connexons), and each hemichannel consists of six protein subunits, the so-called connexins. In mammalian heart, connexin43 (Cx43) is the predominant connexin of the working myocardium and is essential for normal ventricular function. This protein has a short half-life of about 90 min (Beardslee *et al.*, 1998) and is normally found at the intercalated discs of cardiomyocytes. Disturbances in Cx43 expression and distribution may lead to life-threatening arrhythmias. Previously, it was demonstrated that, in patients with congestive heart failure, Cx43 content was down-regulated (Dupont *et al.*, 2001), whereas in patients with left ventricular hypertrophy, the total amount of Cx43 was increased (Kostin *et al.*, 2004). In both patient groups, not only was the overall Cx43 content altered, but also the distribution of Cx43 gap junction channels.

Catecholamines play an important role in many cardiac diseases, such as dilated (DCM) or hypertrophic cardiomyopathy (HCM), and it was shown by others that β-adrenoceptors might participate in hypertrophic responses (Schäfer *et al.*, 2001; Taimor *et al.*, 2004; Zhang *et al.*, 2005). In a previous study our working group evaluated the effect of α- and β-adrenoceptor agonists on Cx43 expression, and we could demonstrate that stimulation of either α- or β-adrenoceptors significantly increased Cx43 mRNA and protein content in rat cardiomyocytes, which was accompanied by a significant rise in gap junction current (Salameh *et al.*, 2006).

The goal of our present study was to investigate the underlying signal transduction pathways that may be involved in β-adrenoceptor dependent changes in Cx43. Because, as mentioned above, Cx43 has a fairly short half-life, an incubation period of 24 h would allow a several-fold turnover of Cx43 proteins. Thus, this time schedule might simulate a long-term or 'chronic' stimulation of β-adrenoceptors, as can be expected in various cardiac diseases. For our studies we used a cell culture model of neonatal rat cardiomyocytes and analysed Cx43 expression and signal transduction pathways using Western blot enzyme-linked immunosorbent assay (ELISA, Tecan, Austria), electromobility shift assay (EMSA) and real-time polymerase chain reaction (PCR) techniques. Moreover, we examined human cardiac biopsies from the left ventricle from either normal hearts (i.e. normal ejection fraction and no hypertrophy) or from patients with congestive heart failure (DCM) (ejection fraction below 35%), or from patients suffering from HCM, and analysed Cx43 expression and distribution in these specimens using Western blot and immunofluorescence techniques.

Methods

Cell culture

Cardiomyocytes were isolated and cultured according to Salameh *et al.* (2004). Briefly, ventricles of newborn Sprague-Dawley rats were digested in collagenase II solution, resuspended in M199 containing 1% fetal calf serum and 10% horse serum (to inhibit fibroblast growth), and seeded in Petri dishes coated with 0.1% gelatin. Medium was changed three times a week. The percentage of non-cardiac cells (fibroblasts, endothelial cells) was below 5% as revealed by specific immunohistology (prolyl-4-hydroxylase; von Willebrand's factor) and did not change during the various treatments. In a first set of experiments, confluent monolayers were exposed to isoprenaline (10^{-10} to 10^{-6} M, each concentration with $n = 10$) for 24 h, and Cx43 protein and Cx43 mRNA expression were investigated. All experiments were performed in the presence of 10 μM nialamide (monoamine oxidase inhibitor) in order to prevent rapid degradation of the catecholamines.

To elucidate the underlying signal transduction pathways in more detail, additional experiments were performed with a concomitant treatment of the cardiomyocytes for 24 h with 1 μM isoprenaline in the presence of either the β-adrenoceptor antagonist propranolol (0.1 μM; Pönicke *et al.*, 2002) or the protein kinase A (PKA) inhibitor (N-[2-(methylamino)ethyl]-5-isoquinolinesulphonamide) (H8; Alexis Biochemicals, San Diego, CA, USA) (2 μM; Engh *et al.*, 1996) or the p38 mitogen-activated protein kinase (MAPK) inhibitor (4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)-1H-imidazole) (SB203580) (Alexis Biochemicals) (10 μM; Goh *et al.*, 1999) or the MEK 1 (MAPK 1 kinase) inhibitor (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one) (PD98059) (Alexis Biochemicals) (10 μM; Warn-Cramer *et al.*, 1998), or the calcineurin-inhibitor cyclosporin A (5 μM; Pönicke *et al.*, 1999). Thereafter, connexin expression was analysed using immunoblotting or real-time PCR as described previously (Salameh *et al.*, 2006) and below.

Moreover, phosphorylated proteins of the MAPK cascade and the transcription factors, activator protein 1 (AP1), CRE-binding protein (CREB) and nuclear factor of activated T-cells (NFATc3), were investigated.

To assess cell viability during the various treatments, a commercial Trypan blue cell viability test (Invitrogen, Karlsruhe, Germany) was used.

Western blots

After 24 h of treatment with isoprenaline with or without the various inhibitors, cells were harvested and lysed at 4°C applying three periods of ultrasound for 10 s each using a low-salt buffer with inhibitors of proteases and phosphatases (10 $\mu g \cdot mL^{-1}$ aprotinin, 10 $\mu g \cdot mL^{-1}$ leupeptin, 10 $\mu g \cdot mL^{-1}$ pepstatin A, 10 nM okadaic acid, 100 μM phenyl arsinoxide, 100 μM cantharidin, 0.1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 20 mM Na_3PO_4, 150 mM NaCl, 2 mM $MgCl_2$, 0.1% Nonidet P40, 1% Triton X-100, 1% sodium dodecyl sulphate (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 30 μg of protein per slot fractionated through a 4% stacking and a 10% running SDS-polyacrylamide gel. Proteins were then transferred to a nitrocellulose membrane by semi-dry blot technique and blocked with 5% low-fat milk at 4°C overnight. Primary antibodies (as described below; also see 'Materials') were applied for 2 h at room temperature, and the following dilutions were used: for Cx43 1:5000, for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Acris, Hiddenhausen, Germany)1:10 000, for NFATc3 1:1000, for c-fos 1:1000, for CREB 1:1000, and for phosphorylated p42/44 and

p38 (Santa Cruz) 1:1000. Thereafter, the blots were washed with phosphate-buffered saline containing: NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 8.3 mM, KH_2PO_4 1.5 mM, pH 7.4 and 0.1% Tween 20, and were incubated with secondary horseradish peroxidase-labelled antibody (Sigma-Aldrich) diluted 1:5000 for 1 h at room temperature. Subsequently, the detection was carried out using the iodophenol/luminol system by application of enhanced chemiluminescence Western blot detection kit from Amersham Pharmacia Biotech. 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, digitized and analysed with BioRad software (BioRad, München, Germany). After background subtraction, gray scale values of the specific signals in the experimental groups were compared with signals of the untreated control cells. All bands were normalized to GAPDH content (assessed by the same method as described above). To calculate the ratio of the phosphorylated slower migrating band and the non-phosphorylated faster migrating band of Cx43, both bands were evaluated separately. The ratio was investigated and the signals of the experimental groups were compared with the signals of the untreated control cells.

The phosphorylated forms of p38 and p42/44 were evaluated in relation to total p38 and total p42/44 proteins (i.e. phosphorylated and non-phosphorylated forms), respectively, and again the signals of the experimental groups were compared with the signals of the untreated 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 standard protocols. After first-strand cDNA was prepared, 1 μL cDNA was mixed with PCR reagents using SYBR Green supermix (BioRad) according to the manufacturer's instruction to make a 25 μL solution, and real-time PCR was carried out using the following primer pairs (Salameh *et al*., 2003):

- Cx43 antisense 5′-TTG TTT CTG TCA CCA GTA AC-3′
  sense 5′-GAT GAG GAA GGA AGA GAA GC-3′
- GAPDH antisense 5′-CCG CCT GCT TCA CCA CCT TCT-3′
  sense 5′-GTC ATC ATC TCC GCC CCT TCC-3′

All samples were collected at the threshold cycle, and the relative amount of Cx43-mRNA in comparison to the mRNA of the housekeeping gene GAPDH was evaluated, according to Livak and Schmittgen (2001), using the $2^{-\Delta\Delta C_t}$ method. Briefly, using this method, the data are presented as the fold in gene expression normalized to the housekeeping gene GAPDH and relative to the untreated control. From the C_t values (defined as the threshold cycle at which the SYBR Green fluorescence exceeds background fluorescence), as automatically determined, for both genes the ΔC_t values were calculated as $C_{t,Cx43} - C_{t,GAPDH}$. These data were analysed according to the following equation:

$$\Delta\Delta C_t = (C_{t,Cx43} - C_{t,GAPDH})_{\text{isoprenaline treatment}} - (C_{t,Cx43} - C_{t,GAPDH})_{\text{no treatment}}. \quad (1)$$

From the $\Delta\Delta C_t$ values, the term $2^{-\Delta\Delta Ct}$ was calculated.

To verify that the quantitative PCR run produced only one band for Cx43 and GAPDH, respectively, with the predicted base pair length (600 bp for Cx43 and 415 bp for GAPDH), the samples were transferred to ethidium bromide-stained 1.5% agarose gels.

Sandwich ELISA

To evaluate the phosphorylated forms of JUN NH_2-terminal kinase (JNK), a detection kit from Cell Signaling (Danvers, MA, USA) was used. Cells grown on Petri dishes were harvested and lysed using the following lysis buffer: 20 mM TRIS (pH 7.5), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 1 $\mu g \cdot mL^{-1}$ leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF).

The cell lysates were added to the microtitre plates coated with the primary antibody against phosphorylated-(Thr183/Tyr185) or total-JNK, respectively, and incubated at 4°C overnight. Thereafter, according to the manufacturer's instructions, the cells were incubated with a secondary detection antibody for 2 h at room temperature, and after several washing steps, with the third horseradish peroxidase-labelled antibody. The dye reaction was carried out using 3,3′5,5′-tetramethylbenzidine, and dye development was evaluated at 450 nm using an ELISA reader (Tecan). The phosphorylated forms of JNK were evaluated in relation to the total-JNK (phosphorylated and non-phosphorylated forms), respectively, and the signals of the experimental groups were compared with the signals of the untreated control cells.

Preparation of nuclei for transcription factor analysis

The preparation of nuclear extracts was carried out using the nuclear extraction kit from Panomics (Fremont, CA, USA). According to the manufacturer's protocol, the cells were grown on Petri dishes and harvested with a buffer containing 10 mM HEPES pH 7.9, 10 mM KCl, 10 mM EDTA, 10 μL 100 mM dithiothreitol (DTT), 10 μL protease inhibitor cocktail and 40 μL 10% Igepal (tert-octylphenoxy poly(oxyethylene)ethanol) (Sigma-Aldrich, Steinheim, Germany). The cells were centrifuged (3 min, 15 000× *g*), and the pellets containing the nuclear fraction were resuspended in the following buffer: 20 mM HEPES pH 7.9, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1.5 μL 100 mM DTT and 1.5 μL protease inhibitor cocktail, and were vigorously agitated for 2 h at 4°C. Afterwards, a second centrifugation step (5 min, 15 000× *g*) was performed, and in the supernatants now containing the nuclear extracts, protein concentration was determined. Furthermore, to test the purity of the extracts, the lactate dehydrogenase (LDH) content (an enzyme found only in the cytoplasm of cells) was measured in the nuclear and cytosolic fractions using the 'Cytotoxicity Detection Kit^{Plus}' from Roche Applied Science (Mannheim, Germany). Thereafter, the nuclear extracts were used for EMSA analysis of AP1 or CREB.

EMSA
To evaluate the transcription factors AP1 and CREB, we used a detection kit from Panomics. According to the manufacturer's protocol, 5 μg of nuclear extract was mixed with the reaction buffer, and the biotinylated-DNA-consensus sequence of either AP1 (5′-CGCTTGATGACTCAGCCGGAA-3′) or CREB (5′-AGAGATTGCCTGACGTCAGAGAGCTAG-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 6% polyacrylamide gel and transferred 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, digitized and analysed as described above.

Patients
The institutional Ethical Committee approved the study and all patients gave written informed consent. The study was conducted according to international guidelines and the declaration of Helsinki. Patients enrolled in this study, underwent coronary angiography, and during heart catheterization, 6–8 myocardial samples from their left ventricular free wall were obtained. Half of the specimens was analysed by our Department of Pathology to confirm the diagnosis of DCM or HCM, which were made by clinical and echocardiographic evaluations; the other samples were used to investigate Cx43 content and distribution by Western blot, PCR and immunohistology. It is important to note that only biopsies of patients without coronary artery stenosis (i.e. without ischaemic cardiomyopathy) and, apart from their cardiac disease, without any other serious diseases (no diabetes mellitus and no renal failure) were analysed. In total, 32 patients were examined: 20 patients with DCM, 6 patients with HCM and 6 control patients (Table 1). The latter ones underwent heart catheterization because of the suspected diagnosis of a restrictive cardiomyopathy, which was excluded by right and left heart catheterization and by our Department of Pathology.

One portion of the acquired biopsies was either processed for Western blot or for real-time PCR in the same manner as described above for the cultured rat cardiomyocytes; another portion was evaluated using indirect immunohistology as described below.

Table 1 Patient data

	Control	*DCM*	*HCM*
Number	6	20	6
Men/women	2/4	15/5	2/4
Age (years)	52 ± 10	53 ± 14	60 ± 9
EF%	67 ± 3	28 ± 5	73 ± 4
β-blocker	3/6	20/20	1/6
ACE inibitor	3/6	15/20	0/6
Calcium antagonist	0/6	0/20	4/6

ACE, angiotensin-converting enzyme; DCM, dilated cardiomyopathy; EF, ejection fraction; HCM, hypertrophic cardiomyopathy.

Immunohistology of human myocardial biopsies
The left ventricular biopsies were fixed with ice-cold methanol, embedded in Karyon F (Merck, Darmstadt, Germany), and indirect immunofluorescence was performed on 5 μm cryosections cut in parallel to the fibre's longitudinal axis. Thereafter, the specimens were permeabilized with Triton-X 100, and to reduce non-specific background, they were blocked with 1% bovine serum albumin in phosphate-buffered saline solution (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 8.3 mM, KH_2PO_4 1.5 mM, pH 7.4) for 30min at room temperature. Primary rabbit anti-Cx43 antibody (1:100) was applied overnight at 4°C. Thereafter, the specimens were exposed to fluorescein isothiocyanate (FITC)-labelled secondary antibody (Sigma-Aldrich) (1:300 for 1 h), subsequently washed with phosphate-buffered saline and embedded in glycerol gallate solution (0.5 mg n-propyl gallate dissolved in 3 mL TRIS-HCl and 7 mL glycerol, pH 9.3). According to the study of Polontchouk *et al*. (2001), only cells cut longitudinally were evaluated. Briefly, the slides were investigated at 1000× magnification using a commercial image analysis system (SigmaScan, Jandel Scientific, Erkrath, Germany) and a Zeiss Axiolab fluorescence microscope (Zeiss, Jena, Germany). Each cell was measured, and the longitudinal as well as the polar plasma membrane length were determined. Thereafter, the length of the immunofluorescence positive membrane (specific fluorescence of the FITC-labelled gap junction proteins) was quantified, and the ratio between positively stained membrane length and plasma membrane length (longitudinal or polar membrane) was calculated. In each group (control, DCM and HCM) 100 cells per slide were analysed in this manner.

Statistical analysis
The concentration-responsecurves were analysed for C_{max}, EC_{50} and Hill slope, and were fitted to a sigmoidal curve using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). For statistical analysis, analysis of variance (ANOVA) was performed, and if ANOVA indicated significant differences ($P < 0.05$), the data were subsequently analysed with the *post hoc* Tukey honestly significant differences (HSD) test, if necessary corrected for multiple comparisons (Bonferroni). For statistical analysis, the software 'Systat for Windows, ver. 11' (Systat, Evanston, IL, USA) was used.

Materials
SB203580, PD98059 and H8 were purchased from Alexis Biochemicals. The polyclonal antibodies (raised in rabbit) against the phosphorylated and non-phosphorylated forms of p38 (product number: sc-7975-R and sc-728), p42/44 (product number: sc-7976-R and sc-153) and the NFATc3 primary antibody (product number: sc-8321) were purchased from Santa Cruz. Polyclonal Cx43 antibody raised in rabbit (product number: C6219) was obtained from Sigma-Aldrich, and monoclonal GAPDH antibody raised in mouse (product number: 5G4 MAb 6C5) was obtained from Acris. Secondary either horseradish peroxidase- or FITC-labelled antibodies were obtained from Sigma-Aldrich. The primers for Cx43 and GAPDH were bought from InViTek (Berlin, Germany). The

cell culture media were purchased from Invitrogen. All other chemicals were obtained from Sigma-Aldrich.

Results

Cell viability

Cell viability was assessed after 24 h of treatment with isoprenaline (1 μM) without or with the various inhibitors demonstrating that there was no significant difference in cell viability among the different experimental groups (control: 98 ± 0.9%; isoprenaline: 97 ± 0.9%; isoprenaline + propranolol: 98 ± 0.6%; isoprenaline + H8: 98 ± 1.2%; isoprenaline + SB203580: 99 ± 0.6%; isoprenaline + PD98059: 98 ± 0.9%; isoprenaline + cyclosporin A: 98 ± 1.2%, $n = 3$).

PCR results of rat cardiomyocytes

Cultured neonatal rat cardiomyocytes were stimulated with increasing concentrations of isoprenaline (10^{-10} to 10^{-6} M) for 24 h, and Cx43 mRNA was analysed using real-time-PCR technique. In these experiments we found a significant concentration-dependent increase in Cx43 mRNA relative to the housekeeping gene GAPDH with a pEC_{50} of 7.75 ± 0.057, R^2=0.97 ($n = 10$) (Figure 1). As this indicated a possible control of Cx43 expression by β-adrenoceptor activation, we tried to elucidate the downstream signalling cascade and possible involvement of p38 and p42/44 MAPK. Therefore, in the next set of experiments, the inhibitors propranolol, SB203580 or PD98059 or H8 or cyclosporin A were administered concomitantly to 1 μM isoprenaline. We found a significant and complete suppression of the isoprenaline-induced increase in Cx43 mRNA by the β-adrenoceptor antagonist, propranolol, by the PKA inhibitor H8, by the p38 MAPK blocker SB203580 and by the p42/44 blocker PD98059 (Figure 2).

Western blot and ELISA results of rat cardiomyocytes

Thereafter, to determine whether the isoprenaline-induced rise in Cx43 mRNA is indeed followed by an enhanced synthesis of Cx43 protein, Western blot studies were performed. In these experiments we also found a significant concentration-dependent increase in Cx43 protein content with a pEC_{50} of 7.92 ± 0.11, R^2=0.89 ($n = 10$), and Hill slope = 1; E_{max} was achieved at 1 μM isoprenaline (Figure 1). GAPDH expression remained unaltered by this treatment. For further investigation of the underlying signalling pathway, we examined the influence of the β-adrenoceptor antagonist propranolol and of inhibitors of possibly relevant kinases on the isoprenaline-induced Cx43 up-regulation. Simultaneous incubation of the cells with isoprenaline together with either propranolol or the p42/44-inhibitor PD98059, or the p38-inhibitor SB203580 or the PKA-inhibitor H8, or the calcineurin-inhibitor cyclosporin A led to a complete inhibition of the isoprenaline-induced rise in Cx43 ($P < 0.05$) (Figure 3, upper part). Additionally, the phosphorylation status of Cx43 was investigated, and it became apparent that isoprenaline led to an enhanced phosphorylation of Cx43, which was inhibited by a concomitant treatment with the above-mentioned inhibitors (Figure 3, lower part). Given

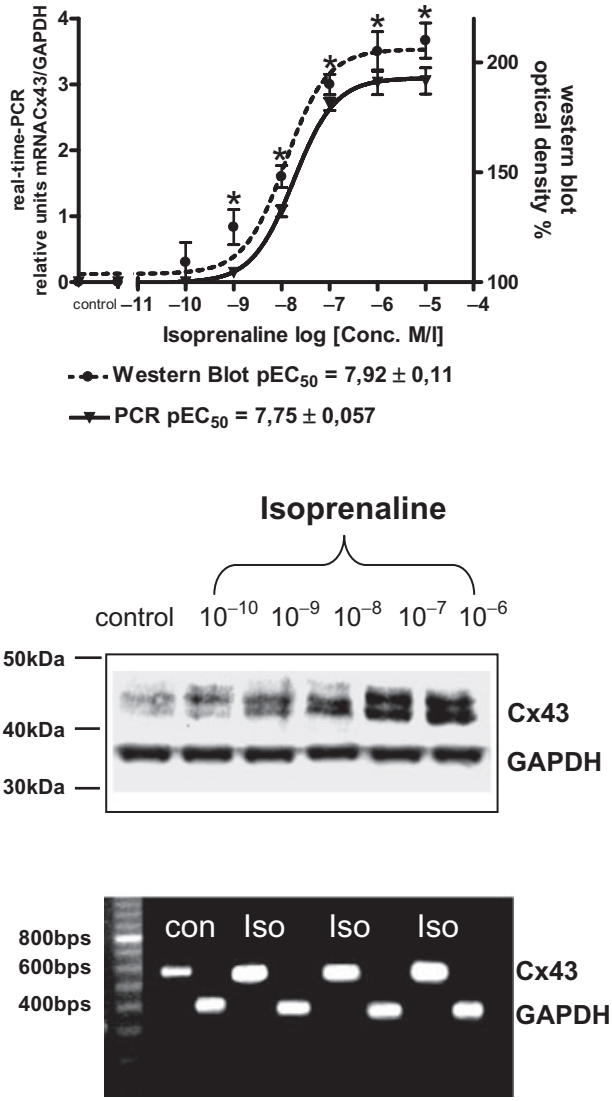

Figure 1 Upper part: up-regulation of connexin43 (Cx43) after stimulation with isoprenaline for 24 h. The figure shows mRNA data from real-time PCR (left Y-axis) and protein data from Western blotting (right Y-axis). *$P < 0.05$, significantly different from untreated baseline values. All data are given as means ± SEM of $n = 10$ experiments. Lower part: original Western blot demonstrating the concentration-dependent increase in Cx43 protein after 24 h stimulation with isoprenaline in relation to GAPDH. Original ethidium bromide-stained agarose gels demonstrating the increase in Cx43 mRNA in relation to GAPDH mRNA after isoprenaline stimulation. Con, control; Iso, isoprenaline 10^{-6} M.

alone, these inhibitors did not affect Cx43 expression (control: 100%; propranolol: 98 ± 11%; SB203580: 90 ± 5%; PD98059: 100 ± 14%; H8: 95 ± 13%; cyclosporin A: 92 ± 4%, $n = 4$). Moreover, these inhibitors, given alone, also did not affect Cx43 phosphorylation (see Figure 3, lower part, 'inhibitor control').

Furthermore, Western blot analysis was carried out on cardiomyocytes stimulated with 1 μM isoprenaline (24 h) for the phosphorylated forms of p38 and p42/44. We found a significant increase in the phosphorylated forms of these two important MAPK proteins after stimulation with isoprenaline.

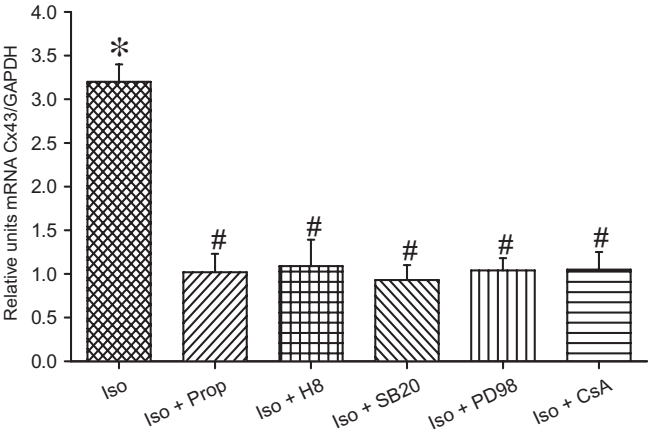

Figure 2 Stimulation of connexin43 (Cx43) mRNA with 1 μM isoprenaline (Iso) for 24 h in the absence or presence of propranolol (Prop), H8, SB203580 (SB20), PD98059 (PD98) or cyclosporin A (CsA). $^{*}P < 0.05$, significantly different from untreated baseline values. $^{\#}P < 0.05$, significantly different from Iso values. All data are given as means ± SEM of $n = 6$ experiments. GAPDH, glyceraldehyde 3-phosphate dehydrogenase (GAPDH); H8, (N-[2-(methylamino) ethyl]-5-isoquinolinesulphonamide); PD98059, (2-(2-amino-3-metho xyphenyl)-4H-1-benzopyran-4-one); SB203580, (4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)-1H-imidazole).

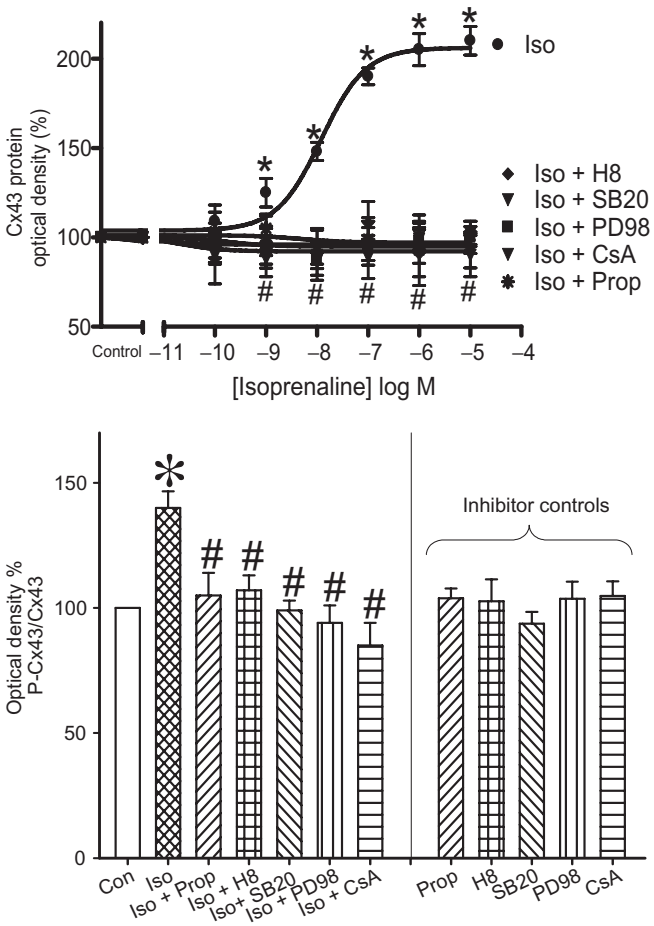

Figure 3 Upper part: inhibition of the isoprenaline (Iso)-induced up-regulation of connexin43 (Cx43) protein by propranolol (Prop), H8, SB203580 (SB20), PD98059 (PD98) or cyclosporin A (CsA). Lower part: inhibition of the Iso-induced up-regulation of the ratio of phosphorylated versus non-phosphorylated Cx43 by Prop, H8, SB20, PD98 or CsA (left side), and inhibitor controls that show no significant influence on basal level of Cx43 phosphorylation (right side). $^{*}P < 0.05$, significantly different from untreated baseline values. $^{\#}P < 0.05$, significantly different from Iso values. All data are given as means ± SEM of $n = 6$ experiments. Con, control; H8, (N-[2-(methylamino)ethyl]-5-isoquinolinesulphonamide); PD98059, (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one); SB2035 80, (4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)-1H-imidazole).

This increased phosphorylation could be blocked by the PKA-inhibitor H8 and by the β-adrenoceptor antagonist propranolol (Figure 4). As Takemoto *et al*. (1999) demonstrated in an *in vivo* study that extracellular signal-regulated kinase (ERK) as well as JNK activity in heart can increase as a result of isoprenaline stimulation, we subsequently studied the phosphorylation status of JNK using the sandwich ELISA technique as described above. We observed a significant increase in phosphorylated JNK in relation to total-JNK. Again, this increase could be inhibited by H8 and propranolol (Figure 4).

Additionally, control experiments have been carried out to examine whether inhibition of one MAPK has an influence on the other two kinases. Incubation of the cardiomyocytes with 1 μM isoprenaline for 24 h without or with SB203580 or PD98059 revealed that these two MAPK inhibitors had no influence on the other two MAPKs (i.e. inhibition of p38 with SB203580 had no influence on the phosphorylation status of p42/44 or JNK) (Figure 5).

Analysis of transcription factors AP1, CREB and NFATc3 (rat cardiomyocytes)

The transcription factor AP1 is involved in many intracellular processes, especially in the MAP-cascade signalling (Frödin and Gammeltoft, 1999). As the results so far suggested involvement of p38 and p42/44 MAPKs, and JNK, and because these MAPKs regulate AP1 and CREB (Markou *et al*., 2004), we wanted to find out whether the intracellular (nuclear) content of these factors was altered by isoprenaline. For that purpose EMSA analysis of nuclear and cytosolic extracts of cardiomyocytes stimulated with 1 μM isoproterenol was performed. First of all, the purity of the extracts was tested using the LDH detection kit from Roche Applied Science, and only nuclear fractions with less than 5% contamination with cytoplasm were used for the subsequent experiments [LDH content: nuclei 0.081 ± 0.018 (arbitrary units) vs. cytosolic fractions 1.99 ± 0.067 (arbitrary units)]. A 24 h stimulation of the cardiomyocytes with isoprenaline resulted in a significant increase in the transcription factors AP1 and CREB in the nuclear fractions, as could be observed by the shift of AP1 and CREB consensus sequence after administration of the nuclear extracts. This shift of the consensus sequence was abolished after 24 h concomitant treatment of the cells with isoprenaline and propranolol or SB203580 or PD98059 (Figure 6).

To confirm the AP1 and CREB-EMSA data, Western blot analysis of nuclear and cytosolic fractions of isoprenaline-stimulated cells was carried out with specific antibodies against c-fos and CREB. Additionally, as the isoprenaline-induced increase in Cx43 was sensitive to cyclosporin A

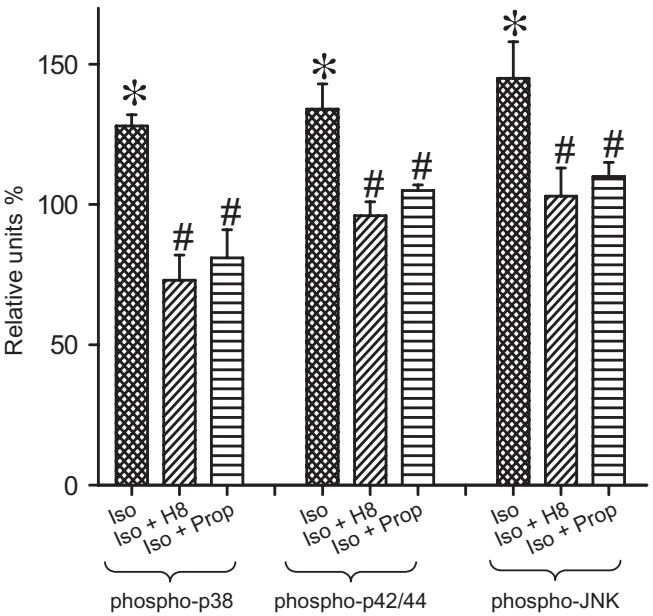

Figure 4 Expression of the phospho-isoforms of p38, p42/44 (measured by Western blot) or JUN NH_2-terminal kinase (JNK) (measured by enzyme-linked immunosorbent assay technique) after stimulation with isoprenaline (Iso) (1 μM) for 24 h in the absence or presence of H8 (2 μM) or propranolol (Prop) (0.1 μM). $^{*}P < 0.05$, significantly different from untreated baseline values. $^{\#}P < 0.05$, significantly different from Iso values. All data are given as means ± SEM of $n = 6$ experiments. H8, (N-[2-(methylamino)ethyl]-5-isoquinolinesulphonamide).

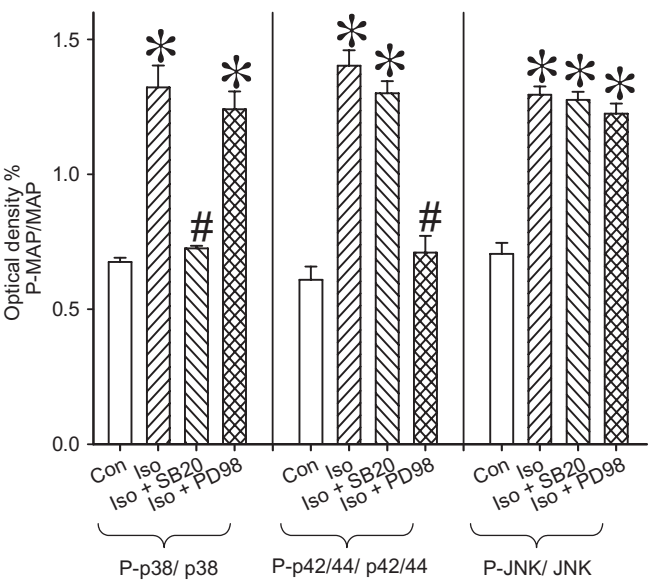

Figure 5 Evaluation of the phosphorylated forms of p38, p42/44 and JUN NH_2-terminal kinase (JNK) in relation to the corresponding total protein (i.e. phosphorylated and non-phosphorylated forms) measured by Western blot, after stimulation with isoprenaline (Iso) for 24 h in the absence or presence of SB203580 (SB20) or PD98059 (PD98). $^{*}P < 0.05$, significantly different from untreated baseline values. $^{\#}P < 0.05$, significantly different from Iso values. All data are given as means ± SEM of $n = 4$ experiments. Con, control; MAP, mitogen-activated protein; P, phospho; PD98059, (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one); SB203580, (4-(4-fluoro phenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)-1H-imidazole).

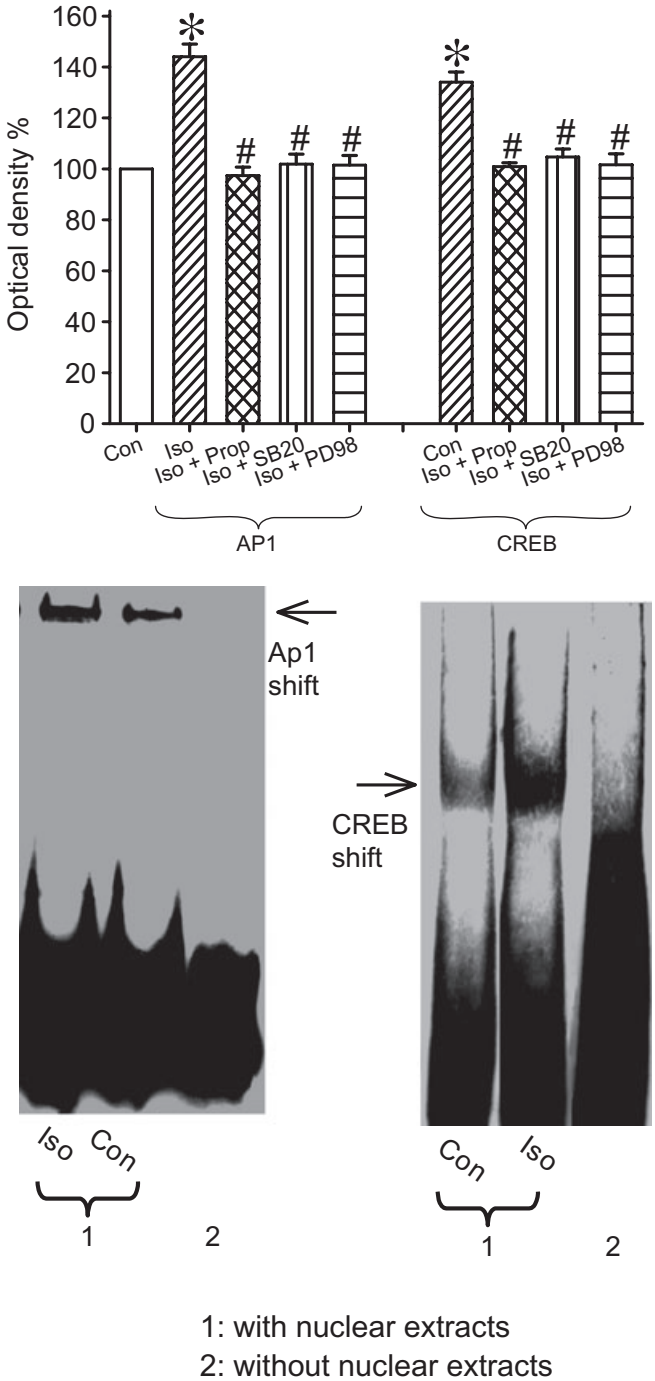

Figure 6 Upper part: electromobility shift assay (EMSA) analysis of the translocation of activator protein 1 (AP1) and CRE-binding protein (CREB) from cytosolic to nuclear fraction after stimulation with 1 μM isoprenaline (Iso) for 24 h in the absence or presence of propranolol (Prop), SB203580 (SB20) or PD98059 (PD98). $^{*}P < 0.05$, significantly different from untreated baseline values. $^{\#}P < 0.05$, significantly different from Iso values. The data are given as means ± SEM from $n = 6$ experiments. Lower part: original EMSA blots, demonstrating AP1 and CREB shift respectively. Con, control; PD98059, (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one); SB203580, (4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)-1H-imidazole).

(Figures 2 and 3), and as cyclosporin A inhibits the phosphatase calcineurin, which dephosphorylates the transcription factor NFATc3 thereby enabling NFATc3 to enter the nucleus, the content of this transcription factor was also analysed by Western blot in the nuclear and cytosolic fractions of isoprenaline-stimulated cells. After isoprenaline treatment, all three transcription factors showed a significant increase in translocation from the cytosol into the nucleus (Figure 7).

Western blot, PCR and immunohistology of human biopsies

Analysis of the Cx43 protein content in human left ventricular biopsies revealed that in patients suffering from HCM, Cx43 content was significantly higher (Cx43 protein: 281 ± 8%) compared with patients without cardiomyopathy (control patients, Cx43 protein: 128 ± 6%). In contrast, in patients with DCM, the Cx43 content was significantly lower (Cx43 protein: 57 ± 3%) compared with control patients. These results were also detectable in the PCR studies: patients with HCM had significantly higher Cx43 mRNA levels and patients with DCM had significantly lower Cx43 mRNA levels than control patients (Figure 8). GAPDH protein and mRNA levels remained unaltered among these three groups.

Besides the total amount of Cx43, its distribution within the cell membrane is also critical to a regular propagation of excitation. Cardiomyocytes from normal hearts usually show an anisotropic distribution of Cx43 gap junction channels with Cx43 concentrated at the cell poles and with only small amounts of Cx43 located at the longitudinal (lateral) cell membrane. As shown in Figure 9, the distribution of immunopositive staining for Cx43 in left ventricular biopsies from control patients yielded a ratio of polar : lateral staining of about 5 (about 50% polar and 10% lateral). In samples from DCM patients, the significant change was a fall in polar staining with unaltered lateral distribution, giving a ratio of about 2.25. Samples from patients with HCM showed increases in both polar and lateral staining (Figure 9), relative to control values with a consequently decreased polar : lateral ratio of about 2.2.

Discussion

As in our previous study, we found here a clear induction of Cx43 protein and mRNA by β-adrenoceptor stimulation, sensitive to inhibition of PKA (Salameh *et al.*, 2006). Thus, we could reproduce our earlier results and corroborate the data of Darrow *et al.* (1996), who found a significant increase in Cx43 after application of dibutyryl-cAMP (db-cAMP) (a membrane-permeable derivative of cAMP, the downstream messenger of β-adrenoceptors). However, in the present study, we further investigated the downstream signal transduction following β-adrenoceptor stimulation with regard to kinases and transcription factors possibly involved in the control of Cx43 expression by β-adrenoceptors.

The present data demonstrated activation of three different MAPKs, that is, p38, p42/44 and JNK, as their respective phosphorylated (active) isoforms were found to be up-regulated in response to β-adrenoceptor stimulation. As

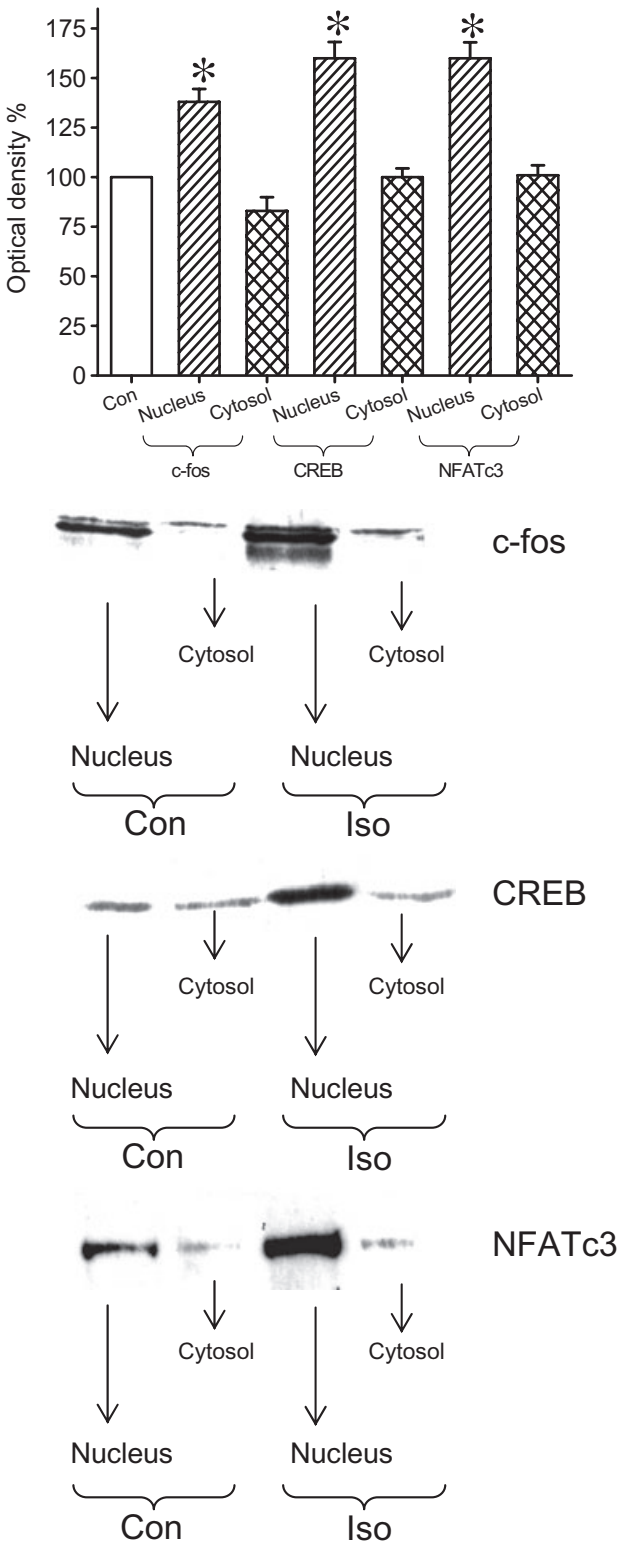

Figure 7 Upper part: Western blot analysis of the translocation of c-fos, CRE-binding protein (CREB) and nuclear factor of activated T-cells (NFATc3) from cytosolic to nuclear fraction after stimulation with 1 μM Iso for 24 h. *$P < 0.05$, significantly different from untreated baseline values. All data are given as means ± SEM of $n = 6$ experiments. Lower part: original Western blots demonstrating the increase in transcription factor content in the nuclei after 24 h stimulation with Iso. Con, control; Iso, isoprenaline 10^{-6} M.

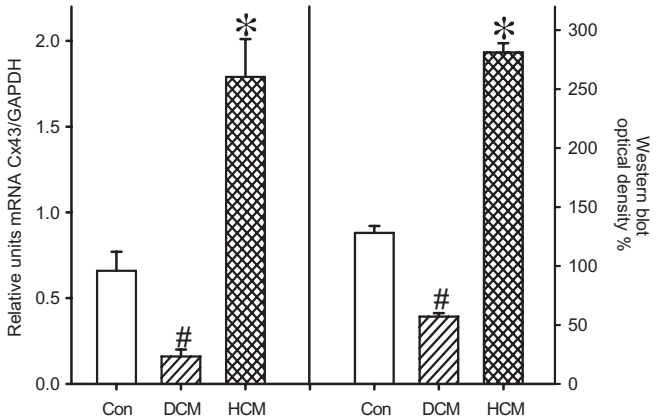

Figure 8 Down-regulation of connexin43 (Cx43) in patients with dilated cardiomyopathy (DCM), and up-regulation of Cx43 in patients with hypertrophic cardiomyopathy (HCM), compared with control patients. The figure shows mRNA data from RT-PCR (left Y-axis) and protein data from Western blotting (right Y-axis). $^{*}P < 0.05$, significantly increased over control. $^{\#}P < 0.05$, significantly decreased over control. All data are given as means ± SEM of $n = 6$ experiments (control and HCM) and of $n = 20$ experiments (DCM). Con, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

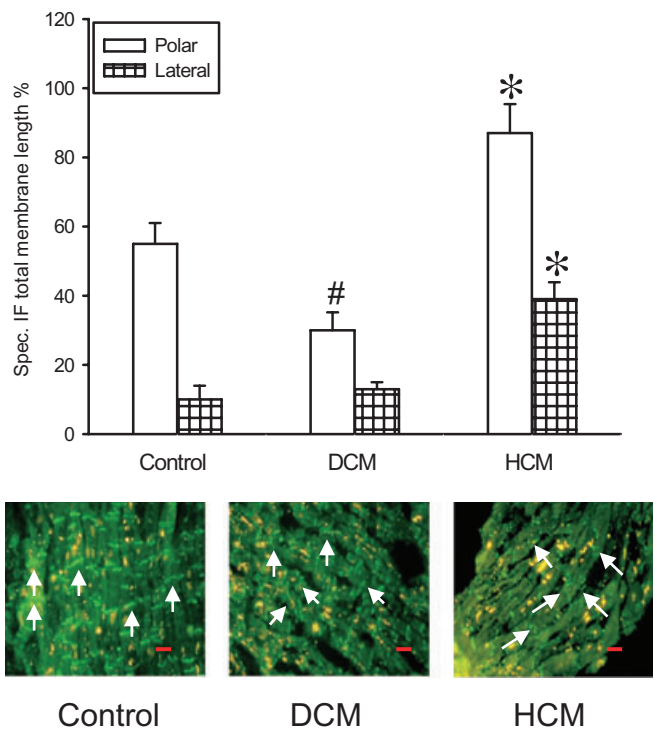

Figure 9 Upper part: reorganization of connexin43 (Cx43) gap junction channels as revealed by immunohistology in cardiomyocytes from patients with dilated cardiomyopathy (DCM) or hypertrophic cardiomyopathy (HCM), compared with control patients. The figure shows the proportion of the total polar membrane length that stained positively for Cx43 [specific immunofluorescence (spec. IF)] and the corresponding proportion of the lateral membrane. $^{*}P < 0.05$, significantly increased over control. $^{\#}P < 0.05$, significantly decreased over control. All data are given as means ± SEM of $n = 6$ experiments (control and HCM) and of $n = 20$ experiments (DCM). Lower part: original specimen of control patients and of patients suffering from DCM or HCM. Spec. IF (green stained Cx43) is indicated by white arrows. The red scale bar represents 20 μm.

propranolol completely abolished the isoprenaline-induced phosphorylation of the three MAPKs, this phosphorylation was mediated by β-adrenoceptor stimulation. Moreover, the present data show that this phosphorylation following β-adrenoceptor stimulation was also mediated by PKA, as it could be completely suppressed by additional H8 treatment. This result was somewhat surprising as it implied that β-adrenoceptor stimulation in the heart activated three signal pathways, p38 MAPK, p42/44 and JNK, all dependent on PKA. However, these results are in agreement with those of Zhang *et al.* (2005), who also found in isoprenaline-infused adult rats *in vivo* a clear dose-dependent phosphorylation and hence activation of all three MAPKs in cardiac tissue. As in our study, the β-adrenoceptor antagonist propranolol eliminated the isoprenaline-induced MAPK phosphorylation. In earlier studies with cardiomyocytes, the three main MAPK pathways were activated following isoprenaline stimulation (Zou *et al.*, 1999; Zheng *et al.*, 2000; Fan *et al.*, 2006; Krishnamurthy *et al.*, 2007). Thus, from these studies and from our results it might be concluded that following long-term (24 h) β-adrenoceptor stimulation and PKA activation, the MAPK cascade is activated, thereby enhancing Cx43 expression.

Our finding that inhibition of any of the three MAPKs (p38, p42/44 or JNK) led to a significant inhibition of Cx43 expression, and that the inhibitors used did not affect the phosphorylation of the other two kinases, is in favour of the view that these MAPKs are activated in parallel and not by a serial mechanism with one of them upstream and the others downstream. Furthermore, this finding may suggest some kind of cooperation among these pathways (but see below). Moreover, isoprenaline induced greater Cx43 phosphorylation, which was inhibited by propranolol, H8 and the MAPK inhibitors, suggesting that isoprenaline-induced phosphorylation may either be a downstream phenomenon or may occur on several levels of the cascade (PKA, MAPK). In this context it is relevant that there are a variety of possible phosphorylation sites known for MAPKs, PKA and other kinases (see Salameh and Dhein, 2005).

In contrast to our results, de Boer *et al.* (2007), although reporting an enhanced conduction velocity in cultured neonatal rat cardiomyocytes after application of 100 nM isoprenaline, did not find an increase in total Cx43 expression (either as protein or as mRNA). This discrepancy is not easy to explain, but different culturing conditions [i.e. different rats (Sprague Dawley vs. Wistar), different serum concentration in culture, different grade of confluence of the cells, different coating of Petri dishes, different activity of catecholamine-degrading enzymes, etc.] might account for the different results. In addition, these authors did not investigate a complete concentration-response curve, so that it is not clear whether they might have seen the same effect but at a somewhat higher concentration, in particular, as they did not use a monoamine oxidase inhibitor, some of the applied isoprenaline might have been degraded.

Assuming that β-adrenoceptor stimulation activates p38, p42/44 and JNK, as indicated by enhanced phosphorylation of these kinases, via a β-adrenoceptor/PKA pathway finally resulting in enhanced Cx43 expression, this should be reflected by translocation of transcription factors typically associated with these cascades such as AP1 and CREB to the

nucleus. The present data did show translocation of AP1 and CREB to the nucleus after isoprenaline stimulation, as indicated by the results of the EMSA experiments. Moreover, this translocation was prevented by propranolol, indicating an involvement of β-adrenoceptors, by SB203580 and by PD98059 indicating an involvement of the p38 MAPK and ERK.

In accordance with our results, Boutillier *et al.* (1992) and Barthel and Loeffler (1995) also found that isoprenaline stimulated AP1 production and binding activity in a neuronal cell line, which was dependent on cAMP and Ca^{2+}. Moreover, stimulation of the β-adrenoceptor/cAMP/PKA pathway enhanced CREB phosphorylation, activation and translocation into the nucleus of cardiomyocytes (Goldspink and Russell, 1996; Müller *et al.*, 2001).

Another point to address is the question whether stimulation of the MAPK cascade is capable of inducing these transcription factors. There are several lines of evidence that transcriptions factors such as AP1 or CREB are enhanced in the nucleus, after activation of the MAPK cascade (Markou *et al.*, 2004; Aggeli *et al.*, 2006). Taken together, these studies and our results are in favour of the hypothesis that, after stimulation of β-adrenoceptors, the MAPK cascade is activated, and as a result transcription factors (AP1 and CREB) translocate into the nucleus of the cardiomyocyte. As binding sites for these transcription factors are present in the Cx43 promoter (Echetebu *et al.*, 1999; Bailey *et al.*, 2002), Cx43 mRNA and protein expression might be enhanced through this pathway.

Besides the mechanisms already mentioned, β-adrenoceptor activation typically results in elevated intracellular Ca^{2+}, which activates the calcineurin pathway leading to enhanced gene expression. We tested whether this cascade might also be involved and found that the calcineurin inhibitor, cyclosporin A, inhibited the isoprenaline-induced increase of Cx43. Because the Cx43 promoter also contains an NFATc3 consensus binding site (Glover *et al.*, 2003), a possible scheme is that increased Ca^{2+} activates the phosphatase calcineurin resulting in a de-phosphorylation of NFATc3, which as de-phospho-NFATc3 can translocate to the nucleus. This scheme is compatible with our finding that the calcineurin-inhibitor cyclosporin A suppressed isoprenaline-induced Cx43 expression and that NFATc3 translocated into the nucleus after isoprenaline stimulation.

As any one of the signal transduction inhibitors (SB203580, PD98059 and cyclosporin A) totally inhibited the isoprenaline-induced increase in Cx43 expression, it would appear that there was a cooperativity among the three pathways or among the transcription factors.

In order to assess the possibility of up-regulated cardiac Cx43 in human disease, we examined cardiac biopsies from patients with HCM. We showed for the first time that in these biopsies, amounts of Cx43 protein and mRNA were increased, relative to those in control biopsies. There was also a rearrangement of Cx43 within the cell membrane, such that more of this connexin was found at the lateral border of cardiomyocytes from HCM hearts than in cardiomyocytes from control hearts. These results fit well with a histological (but not quantitative biochemical) study of Sepp *et al.* (1996), who found that in hearts of patients with HCM, Cx43 was no longer confined to the intercalated discs (cell pole) but instead showed varying degrees of dispersion over the surface of the cardiomyocytes. In a transgenic rabbit model of HCM, Cx43 density in ventricles of transgenic rabbits was significantly increased compared with wild type and was associated with an enhanced vulnerability (Ripplinger *et al.*, 2007). Thus, enhanced Cx43 levels and altered localization may contribute to the known dysrhythmogenicity of HCM.

In contrast to the hypertrophic heart and to the cell culture model, ventricular biopsies from patients with DCM showed decreased total Cx43 protein and mRNA content compared with control hearts. The distribution of Cx43 was again altered, this time with a greater loss of the polar staining. Several other authors have reported on a down-regulation and an altered Cx43 distribution in DCM (Kaprielian *et al.*, 1998; Dupont *et al.*, 2001; Kostin *et al.*, 2003). As both DCM and HCM are associated with life-threatening ventricular dysrhythmias, it is conceivable that changes in the gap junction protein Cx43, added to other factors such as fibrosis, contribute to the enhanced dysrhythmogenicity and contractile dysfunction of these hearts.

In conclusion, long-term (24 h) β-adrenoceptor stimulation can activate MAPK pathways, including p38, p42/44 and JNK as well as the calcineurin pathway in a PKA-dependent manner, resulting in enhanced synthesis of the gap junction protein Cx43. This mechanism might contribute to the changes in cardiac gap junctions seen in cardiac diseases.

References

Aggeli IKS, Gaitanaki C, Beis I (2006). Involvement of JNKs and p38-MAPK/MSK1 pathways in H_2O_2-induced upregulation of heme oxygenase-1 mRNA in H9c2 cells. *Cell Signal* **18**: 1801–1812.

Bailey J, Phillips RJ, Pollard AJ, Gilmore K, Robson SC, Europe-Finner GN (2002). Characterization and functional analysis of cAMP response element modulator protein and activating transcription factor 2 (ATF2) isoforms in the human myometrium during pregnancy and labor: identification of a novel ATF2 species with potent transactivation properties. *J Clin Endocrinol Metab* **87**: 1717–1728.

Barthel F, Loeffler JP (1995). β_2-adrenoreceptors stimulate c-fos transcription through multiple cyclic AMP- and Ca^{2+} -responsive elements in cerebellar granular neurons. *J Neurochem* **64**: 41–51.

Beardslee MA, Laing JG, Beyer EC, Saffitz JE (1998). Rapid turnover of connexin43 in the adult rat heart. *Circ Res* **83**: 629–653.

de Boer TP, van Rijen HV, Van der Heyden MA, Kok B, Opthof T, Vos MA *et al.* (2007). Beta-, not alpha-adrenergic stimulation enhances conduction velocity in cultures of neonatal cardiomyocytes. *Circ J* **71**: 973–981.

Boutillier AL, Barthel F, Robertsll JL, Loeffler JP (1992). β-adrenergic stimulation of cFOS via protein kinase A is mediated by cAMP regulatory element binding protein (CREB)-dependent and tissue-specific CREB-independent mechanisms in corticotrope cells. *J Biol Chem* **267**: 23520–23526.

Darrow BJ, Fast VG, Kléber AG, Beyer EC, Saffitz JE (1996). Functional and structural assessment of intercellular communication. Increased conduction velocity and enhanced connexin expression in dibutyryl cAMP-treated cultured cardiac myocytes. *Circ Res* **79**: 174–183.

Dupont E, Matsushita T, Kaba RA, Vozzi C, Coppen SR, Khan N *et al.* (2001). Altered connexin expression in human congestive heart failure. *J Mol Cell Cardiol* **33**: 359–371.

Echetebu CO, Ali M, Izban MG, MacKay L, Garfield RE (1999). Localization of regulatory protein binding sites in the proximal region of human myometrial connexin 43 gene. *Mol Hum Reprod* **5**: 757–766.

Engh RA, Girod A, Kintel V, Hubert R, Bossemeyer D (1996). Crystal structures of catalytic subunit of cAMP-dependent protein kinase in complex with isoquinolinesulfonyl preotein kinase inhibitors H7, H8, and H89. Structural implications for selectivity. *J Biol Chem* **271**: 26157–26164.

Fan GC, Yuan Q, Song G, Wang Y, Chen G, Qian J *et al*. (2006). Small heat-shock protein Hsp20 attenuates beta-agonist-mediated cardiac remodeling through apoptosis signal-regulating kinase 1. *Circ Res* **99**: 1233–1242.

Frödin M, Gammeltoft S (1999). Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. *Mol Cell Endocrinol* **151**: 65–77.

Glover D, Little JB, Lavin MF, Gueven N (2003). Low dose ionizing radiation-induced activation of connexin 43 expression. *Int J Radiat Biol* **79**: 955–964.

Goh KC, Haque SJ, Williams BRG (1999). p38 MAP kinase is required for STAT1 serine phosphorylation and transcriptional activation induced by interferons. *EMBO J* **18**: 5601–5608.

Goldspink PH, Russell B (1996). Physiological role of phosphorylation of the cyclic AMP response element binding protein in rat cardiac nuclei. *Cell Tissue Res* **285**: 379–385.

Kaprielian RR, Gunning M, Dupont E, Sheppard MN, Rothery SM, Underwood R *et al*. (1998). Downregulation of immunodetectable connexin43 and decreased gap junction size in the pathogenesis of chronic hibernation in the human left ventricle. *Circulation* **97**: 651–660.

Kostin S, Dammer S, Hein S, Klövekorn WP, Bauer EP, Schaper J (2004). Connexin 43 expression and distribution in compensated and decompensated cardiac hypertrophy in patients with aortic stenosis. *Cardiovasc Res* **62**: 426–436.

Kostin S, Rieger M, Dammer S, Hein S, Richter M, Klövekorn WP *et al*. (2003). Gap junction remodeling and altered connexin43 expression in the failing human heart. *Mol Cell Biochem* **242**: 135–144.

Krishnamurthy P, Subramanian V, Singh M, Singh K (2007). Beta1 integrins modulate beta-adrenergic receptor-stimulated cardiac myocyte apoptosis and myocardial remodeling. *Hypertension* **49**: 865–872.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$t. method. *Methods* **25**: 402–408.

Markou T, Hadzopoulou-Cladaras M, Lazou A (2004). Phenylephrine induces activation of CREB in adult rat cardiac myocytes through MSK1 and PKA signaling pathways. *J Mol Cell Cardiol* **37**: 1001–1011.

Müller FU, Boknik P, Knapp J, Linck B, Lüss H, Neumann J *et al*. (2001). Activation and inactivation of cAMP-response element-mediated gene transcription in cardiac myocytes. *Cardiovasc Res* **52**: 95–102.

Pönicke K, Heinroth-Hoffmann I, Becker K, Osten B, Brodde OE (1999). Gq/11-coupled receptors and protein synthesis in rat cardiomyocytes: role of Gi proteins and protein kinase C-isoenzymes. *Naunyn Schmiedebergs Arch Pharmacol* **360**: 301–308.

Pönicke K, Heinroth-Hoffmann I, Brodde OE (2002). Differential effects of bucindolol and carvedilol on noradenaline-induced hypertrophic response in ventricular cardiomyocytes of adult rats. *J Pharmacol Exp Ther* **301**: 71–76.

Polontchouk L, Haefliger J-A, Berit Ebelt B, Schaefer T, Stuhlmann D, Mehlhorn U *et al*. (2001). Effects of chronic atrial fibrillation on gap junction distribution in human and rat atria. *J Am Coll Cardiol* **38**: 883–891.

Ripplinger CM, Li W, Hadley J, Chen J, Rothenberg F, Lombardi R *et al*. (2007). Enhanced transmural fiber rotation and connexin 43 heterogeneity are associated with an increased upper limit of vulnerability in a transgenic rabbit model of human hypertrophic cardiomyopathy. *Circ Res* **101**: 1049–1057.

Salameh A, Dhein S (2005). Pharmacology of gap junctions. New pharmacological targets for treatment of arrhythmia, seizure and cancer? *Biochim Biophys Acta* **1719**: 36–58.

Salameh A, Frenzel C, Boldt A, Rassler B, Glawe I, Schulte J *et al*. (2006). Subchronic alpha- and beta-adrenergic regulation of cardiac gap junction protein expression. *FASEB J* **20**: 365–367.

Salameh A, Polontchouk L, Dhein S, Hagendorff A, Pfeiffer D (2003). Chronic regulation of the expression of the gap junction protein connexin 43 in transfected HeLa cells. *Naunyn Schmiedebergs Arch Pharmacol* **368**: 33–40.

Salameh A, Schneider P, Mühlberg K, Hagendorff A, Dhein S, Pfeiffer D (2004). Chronic regulation of the expression of gap junction proteins connexin40, connexin43, and connexin 45 in neonatal rat cardiomyocytes. *Eur J Pharmacol* **503**: 9–16.

Schäfer M, Pönicke K, Heinroth-Hoffmann I, Brodde OE, Piper HM, Schlüter KD (2001). Beta-adrenoceptor stimulation attenuates the hypertrophic effect of alpha-adrenoceptor stimulation in adult rat ventricular cardiomyocytes. *J Am Coll Cardiol* **37**: 300–307.

Sepp R, Severs NJ, Gourdie RG (1996). Altered patterns of cardiac intercellular junction distribution in hypertrophic cardiomyopathy. *Heart* **76**: 412–417.

Taimor G, Schlüter KD, Best P, Helmig S, Piper HM (2004). Transcription activator protein 1 mediates alpha- but not beta-adrenergic hypertrophic growth responses in adult cardiomyocytes. *Am J Physiol Heart Circ Physiol* **286**: H2369–H2375.

Takemoto Y, Yoshiyama M, Takeuchi K, Omura T, Komatsu R, Izumi Y *et al*. (1999). Increased JNK, AP-1 and NF-κB DNA binding activities in isoproterenol-induced cardiac remodeling. *J Mol Cell Cardiol* **31**: 2017–2030.

Warn-Cramer BJ, Cottrell GT, Burt JM, Lau AF (1998). Regulation of connexin-43 gap junctional intercellular communication by mitogen-activated protein kinase. *J Biol Chem* **273**: 9188–9196.

Zhang GX, Kimura S, Nishiyama A, Shokoji T, Rahman M, Yao L *et al*. (2005). Cardiac oxidative stress in acute and chronic isoproterenol-infused rats. *Cardiovasc Res* **65**: 230–238.

Zheng M, Zhang SJ, Zhu WZ, Ziman B, Kobilka BK, Xiao RP (2000). Beta 2-adrenergic receptor-induced p38 MAPK activation is mediated by protein kinase A rather than by Gi or gbeta gamma in adult mouse cardiomyocytes. *J Biol Chem* **275**: 40635–40640.

Zou Y, Komuro I, Yamazaki T, Kudoh S, Uozumi H, Kadowaki T *et al*. (1999). Both Gs and Gi proteins are critically involved in isoproterenol-induced cardiomyocyte hypertrophy. *J Biol Chem* **274**: 9760–9770.