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Signal Transduction and Transcriptional Control of Cardiac Connexin43 Up-Regulation after α_1-Adrenoceptor Stimulation

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ABSTRACT

Syncytial behavior of cardiac tissue is mainly controlled by the expression of cardiac gap junction proteins, and of these, connexin43 (Cx43) represents the predominant connexin in the working myocardium. Because the α_1-adrenoceptor is involved in many cardiac diseases, the following experiments were performed to clarify the pathway whereby α_1-adrenoceptor stimulation may control Cx43 expression. Cultured neonatal rat cardiomyocytes were stimulated with phenylephrine for 24 h, and Cx43 expression was investigated. Moreover, we investigated activation of p38 mitogenic-activated protein kinase (MAPK), p42/44-MAPK, and c-JUN NH_2-terminal kinase (JNK) by phosphospecific enzyme-linked immunosorbent assay and nuclear translocation of the transcription factors c-fos and activator protein 1 (AP1). For verification of our results, a Cx43-promoter-enhanced green fluorescent protein (EGFP) construct using the complete promoter [2771 base pairs (bp)] or fragments (0–2421 bp) with EGFP under control of the Cx43 promoter was transfected into cardiomyocytes, and fluorescence intensity was investigated. Phenylephrine exposure caused approximately 2-fold up-regulation of Cx43 protein with an EC_{50} of approximately 5 nM, which was significantly inhibited by bisindolylmaleimide I [protein kinase C (PKC) inhibitor], 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole (SB203580; p38 inhibitor), or 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD98059; p42/44 inhibitor). Similar findings were obtained for Cx43 mRNA. Furthermore, Cx43 up-regulation was accompanied by phosphorylation of p38, p42/44, and JNK. Moreover, we found translocation of c-fos and AP1 to the nucleus. Phenylephrine stimulation of Cx43-promoter EGFP-transfected cardiomyocytes significantly increased fluorescence, depending on the length of promoter fragments. A 91-bp fragment containing the first AP1 binding site produced approximately 50% of the fluorescence intensity of the complete promoter. Therefore, we conclude that α_1-adrenoceptor stimulation up-regulates cardiac Cx43 expression via a PKC p38- and p42/44 MAPK-regulated pathway, possibly involving AP1.

Intercellular communication is an important feature of organization within many kinds of tissue. Gap junction channels form the basis of direct intercellular communication. These channels allow electrical and metabolic coupling between neighboring cells. One complete gap junction channel is composed of two hemichannels (connexons), and each hemichannel consists of six protein subunits, the so-called connexins. A connexin has four transmembrane domains, two extracellular loops, and the N and C terminus at the cytoplasmic side of the cell. The C terminus is the variant part of a connexin and differs in length and amino acid sequence between the various connexin isoforms. Moreover, it is known that the C terminus contains consensus sequences that are susceptible to a number of protein kinases such as protein kinase A, protein kinase B, protein kinase C (PKC), protein kinase G, and mitogen-activated protein kinases (MAPKs) (Lampe 1994, Kwak et al., 1995; TenBroek et al., 2001; Polontchouk et al., 2002).

In the mammalian heart, cardiomyocytes mainly express

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ABBREVIATIONS: PKC, protein kinase C; MAPK, mitogenic-activated protein kinase; Cx43, connexin43; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; AP1, activator protein 1; BIM I, bisindolylmaleimide I; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole; PD98059, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; C_t, threshold cycle; JNK, c-JUN NH_2-terminal kinase; LDH, lactate dehydrogenase; TIS, transcription initiation site; EGFP, enhanced green fluorescent protein; bp, base pairs; ERK, extracellular signal-regulated kinase.

connexin40, connexin43 (Cx43), and connexin45. It has been shown that, in the working myocardium, Cx43 is the predominantly expressed connexin, whereas connexin40 is the dominant connexin in the atria and in the conduction system. Connexin45 is found in embryonic stages of the heart and in small amounts also in the conduction system (Davis et al., 1995). Cx43, which is essential for normal ventricular function, has a short half-life of approximately 90 min (Beardslee et al., 1998), and it is normally found at the intercalated disks of cardiomyocytes. Disturbances in Cx43 expression and distribution may lead to life-threatening arrhythmias. It has been shown by several authors that, in patients suffering from congestive heart failure, Cx43 content is 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 patients groups, not only was the overall Cx43 content altered, but the distribution of Cx43 gap junction channels changed as well. Because catecholamines play an important role in many cardiac diseases such as dilated or hypertrophic cardiomyopathy, and because it was shown by others that α-adrenoceptors may participate in hypertrophic responses (Pönicke et al., 2001), the goal of our study was to evaluate whether stimulation of α-adrenoceptors by incubation with the α_1-adrenoceptor agonist phenylephrine might alter Cx43 expression and to investigate which signal transduction pathways may be involved in an α-adrenoceptor-dependent change in Cx43. Because, as mentioned above, Cx43 has a short half-life, an incubation period of 24 h was expected to allow a severalfold turnover of Cx43 proteins; therefore, we used an incubation time of 24 h to assess changes in Cx43. For that purpose, we used a cell culture model of neonatal rat cardiomyocytes and analyzed Cx43 expression and signal transduction pathways using Western blot, electrophoretic mobility shift assay (EMSA), and real-time polymerase chain reaction (PCR) techniques. Moreover, we transfected the Cx43-promoter or promoter fragments into cardiomyocytes to investigate whether the different transcription factors [c-fos and activator protein 1 (AP1)] involved in α-adrenergic effects might also play a role on Cx43 expression.

Materials and Methods

Cell Culture. Cardiomyocytes were isolated and cultured according to methods described by Polontchouk et al. (2002). In brief, ventricles of newborn Wistar rats were digested in collagenase solution, centrifuged, and, after a preplating period to remove noncardiac cells, resuspended in M199 medium containing 2 mM L-glutamine, 100 mg/ml streptomycin and penicillin, 1% fetal calf serum, and 10% horse serum (to inhibit fibroblast growth). The cells were seeded in Petri dishes coated with 0.1% gelatin, and medium was changed three times a week. The percentage of noncardiac cells (fibroblasts and endothelia cells) was <5%, as revealed by specific immunohistology (prolyl-4-hydroxylase; von Willebrand's factor), and did not change during phenylephrine treatment. In the first set of experiments, confluent monolayers were exposed to phenylephrine [3-(1-hydroxy-2-methylamino-ethyl)phenol] (10^{-10}–10^{-6} M, each concentration with $n = 10$) for 24 h, and Cx43 protein and Cx43 mRNA expression was investigated. To elucidate signal transduction pathways in more detail, we performed additional experiments exposing the cells to phenylephrine (1 μM) in the presence of the α_1-adrenoceptor blocker prazosin ([4-(4-amino-6,7-dimethoxy-quinazoline-2-yl) piperazine-1-yl]-(2-furyl)methanone) (0.1 μM; Fuller et al., 1991), the PKC inhibitor bisindolylmaleimide I (BIM I; 3-(*N*-[dimethylamino]propyl-3-indolyl)-4-(3-indolyl)maleimide) (5 μM; Ruf et al., 2002), the p38 inhibitor SB203580 (10 μM; Goh et al., 1999), or the mitogen-activated protein kinase kinase 1 inhibitor PD98059 (10 μM) (Warn-Cramer et al., 1998). Each of the inhibitors was administered concomitantly with phenylephrine for 24 h.

Thereafter, connexin expression was analyzed 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 AP1 and c-fos were investigated.

For investigation of Cx43 mRNA, cells were harvested using TRIzol (Invitrogen, Carlsbad, CA), and cDNA was synthesized by reverse transcription using 1 μg of total RNA. Real-time PCR was done with primer pairs for Cx43 or the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (see below).

Western Blots. In brief, after 24-h treatment, cells were harvested and lysed at 4°C, applying three strikes of ultrasound for 10 s 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 nM okadaic acid, 100 μM phenylarsinoxide, 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 P-40, 1% Triton X-100, 1% SDS, and 10% glycerol). Total protein concentration was determined using standard protocols. Thereafter, whole-cell lysates were mixed with gel-loading buffer, according to Laemmli following classic protocols, and for electrophoresis, 30 μg of protein per slot was fractionated through a 4% stacking and a 10% running SDS-polyacrylamide gel. Proteins were then transferred onto a nitrocellulose membrane by semidry 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 h at room temperature, and the following dilutions were used: Cx43, 1:2000; GAPDH, 1:180,000; phosphorylated p42/44 and p38, 1:1000; and c-fos, 1:2000. Thereafter, the blots were washed with phosphate-buffered saline containing 137 mM NaCl, 2.7 mM KCl, 8.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, and 0.1% Tween 20 and were incubated with secondary horseradish peroxidase-labeled antibody diluted 1:10,000 for 1 h at room temperature. In subsequent experiments, the detection was carried out using the iodophenol/luminol system by application of ECL Western blot detection kit from GE Healthcare (Chalfont St. Giles, UK). The blots were incubated for 60 s (according to the manufacturer's instructions) with the reaction mixture and then exposed to X-ray film to detect chemiluminescence. The specific bands were imaged on a scanner, digitized, and analyzed with Bio-Rad software (Bio-Rad, 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). The phosphorylated forms of p38 and p42/44 were evaluated in relation to total p38 and total p42/44 proteins (i.e., phosphorylated and nonphosphorylated forms), respectively, and again the signals of the experimental groups were compared to the signals of the untreated control cells.

Reverse Transcription and PCR Amplification. RNA was isolated using TRIzol (Gibco BRL, Germany). Thereafter, RNA was reverse transcribed from 1 μg of total RNA with random hexamers to generate first-strand cDNA using standard protocols. After first-strand cDNA was prepared, 1 μl of cDNA was mixed with PCR reagents using SYBR Green SuperMix (Bio-Rad) according to the manufacturer's instructions to make a 25-μl solution, and real-time PCR was done using the following primer pairs (Salameh et al., 2003): Cx43 antisense, 5′-TTG TTT CTG TCA CCA GTA AC-3′ and sense, 5′-GAT GAG GAA GGA AGA GAA GC-3′; and GAPDH antisense, 5′-CCG CCT GCT TCA CCA CCT TCT-3′ and sense, 5′-GTC ATC ATC TCC GCC CCT TCC-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, according to Livak and Schmittgen (2001), using the

$2^{-\Delta\Delta C}{}_t$ method. In brief, 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 threshold C_t values (defined as the threshold cycle at which the SYBR Green fluorescence exceeds background fluorescence) as automatically determined, the ΔC_t values were calculated as $C_{t,Cx43} - C_{t,GAPDH}$ for both genes. These data were analyzed according to the following equation: $\Delta\Delta C_t = (C_{t,Cx43} - C_{t,GAPDH})_{phenylephrine\text{-}treatment} - (C_{t,Cx43} - C_{t,GAPDH})_{no\ treatment}$.

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

Sandwich Enzyme-Linked Immunosorbent Assay. To evaluate the phosphorylated forms of c-JUN NH_2-terminal kinase (JNK), a detection kit from Cell Signaling Technology Inc. (Danvers, MA) 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 EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride.

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

Preparation of Nuclei for Western Blot and EMSA. The preparation of nuclear extracts was carried out using the nuclear extraction kit from Panomics (Fremont, CA). 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, 10 μl protease inhibitor cocktail, and 40 μl 10% Igepal [*tert*-octylphenoxy poly(oxyethylene)ethanol]. The cells were centrifuged (15,000*g* for 3 min), and the pellets containing the nuclear fraction were resuspended in 20 mM HEPES, pH 7.9, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1.5 μl 100 mM dithiothreitol, and 1.5 μl protease inhibitor cocktail and then vigorously agitated for 2 h at 4°C. Afterward, a second centrifugation step (15,000*g* for 5 min) 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 either used for EMSAs or for Western blot analysis of c-fos content.

EMSA. To evaluate the transcription factor AP1, we also 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 AP1 (5′-CGCTTGATGACTCAGCCGGAA-3′) [poly(dI-dC)] was added to block nonspecific binding). After a 30-min incubation at room temperature, the probes were fractionated through a nondenaturing 6% polyacrylamide gel and transferred onto a nylon membrane using the wet-blot technique. Detection of the specific bands was carried out using horseradish peroxidase-labeled 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 analyzed as described above.

Cx43 Promoter and Transfection Experiments. To establish whether stimulation of the cardiomyocytes with phenylephrine leads to a direct stimulation of the Cx43 promoter, the complete human Cx43-promoter sequence (according to Geimonen et al., 1996) as well as fragments of distinct length [starting at the transcription initiation site (TIS) and extending upstream to −91, −431, −781, −1241, −1511, −1715, −2005, and −2421] were cloned into the pIRES2-enhanced green fluorescent protein (EGFP) vector (Clontech, Mountain View, CA) using the technique described by De Leon et al. (1994), so that EGFP was under control of the Cx43 promoter. The complete promoter sequence served as a positive control, and for negative controls (= autofluorescence of the cells), the pIRES2-EGFP vector without promoter was used. The Cx43 promoter and the pIRES2-EGFP vectors were a generous gift from Dr. A. Schubert (Fraunhofer Institut, Leipzig, Germany).

Neonatal cardiomyocytes were prepared according to the above protocol, and, at day 2, they were transfected with the pIRES2-EGFP vectors containing the various promoter fragments, using Lipofectamine 2000 reagent (Invitrogen, Karlsruhe, Germany). After reaching confluence, the cells were stimulated with 1 μM phenylephrine for 24 h, thereafter treated with ice-cold methanol for fixation, mounted on object slides, and green fluorescence was visualized using a Zeiss microscope with 1000× magnification. For each experiment, 300 cells were measured, and the number of fluorescent cells as well as the fluorescence intensity were assessed with Adobe Photoshop 3.0 software. After background subtraction, the fluorescence values of the cells containing the promoter fragments within the pIRES2-EGFP vector were related to the cells containing the complete promoter sequence (positive control = 100%).

Materials. SB203580, PD98059, and BIM I were purchased from Alexis Biochemicals (San Diego, CA). The polyclonal antibodies (raised in rabbit) against the phosphorylated and nonphosphorylated forms of p38 (product nos. sc-7975-R and sc-728), p42/44 (product nos. sc-7976-R and sc-153), and c-fos (product no. sc-52) were purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Polyclonal Cx43 antibody raised in rabbit (product no. C6219) was obtained from Sigma-Aldrich (Steinheim, Germany), and monoclonal GAPDH antibody raised in mouse (product no. 5G4 MAb 6C5) was from Acris (Hiddenhausen, Germany). 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.

Statistical Analysis. The concentration-response curves were analyzed for C_{max}, EC_{50}, and Hill slope and were fitted to a sigmoidal curve using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

For statistical analysis, analysis of variance was performed, and if analysis of variance indicated significant differences ($p < 0.05$), the data were additionally analyzed with the Tukey's honest significant difference test and corrected for multiple comparisons if necessary. For statistical analysis, the software Systat for Windows, version 11 (Systat Inc., Evanston, IL) was used.

Results

Western Blot Results. Stimulation of cultured neonatal rat cardiomyocytes for 24 h with increasing concentrations of phenylephrine (10^{-10}–10^{-6} M) led to a significant ($p < 0.05$) concentration-dependent increase in Cx43 protein content with a pEC_{50} of 8.26 ± 0.08 (~5.5 nM), $R^2 = 0.94$ ($n = 10$), and Hill slope = 1, and E_{max} was achieved at 1 μM phenylephrine (Fig. 1). GAPDH expression remained unaltered by this treatment. For further investigation of the underlying signaling pathway, we examined the influence of inhibitors of possibly relevant kinases on the phenylephrine-induced Cx43 up-regulation. Simultaneous incubation of the cells with phenylephrine and with either the p42/44-inhibitor PD98059 or the PKC-inhibitor BIM I led to a complete inhibition of the phenylephrine-induced rise in Cx43 ($p < 0.05$). The p38 blocker SB203580 also significantly inhibited the

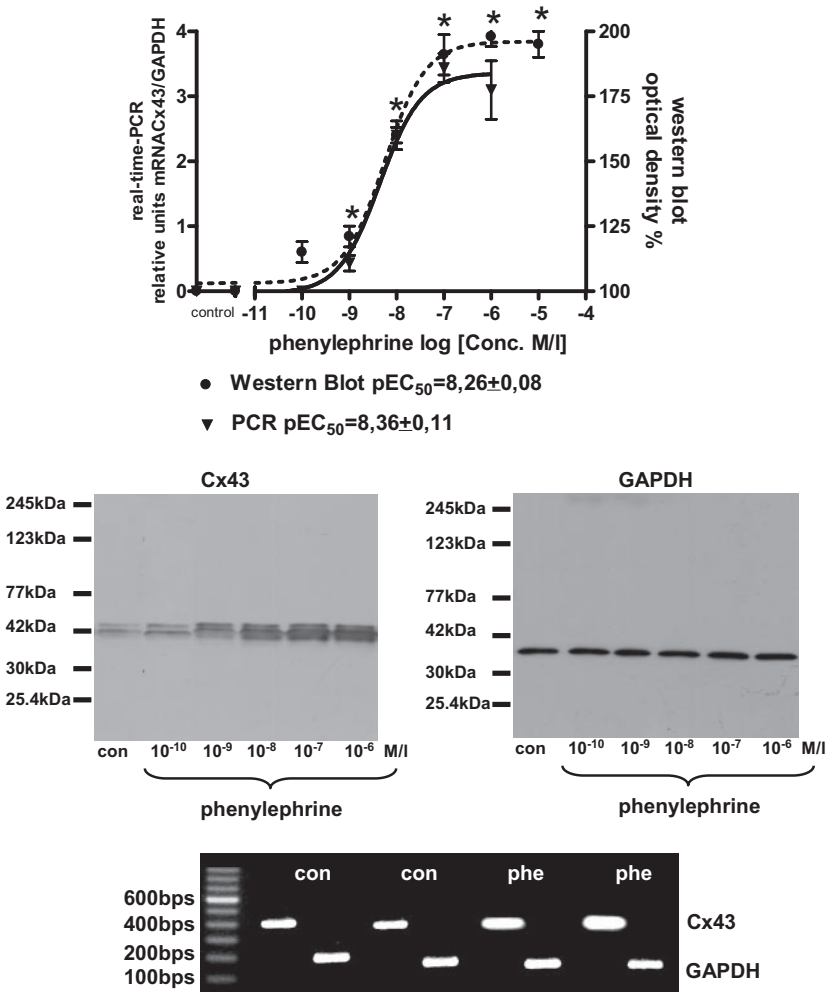

Fig. 1. top, up-regulation of Cx43 after stimulation with phenylephrine for 24 h. The figure shows protein data from Western blotting (right *y*-axis) and mRNA data from real-time PCR (left *y*-axis). Significance versus untreated baseline is indicated by an asterisk ($p < 0.05$). All data are given as means ± S.E.M. of $n = 10$ experiments. bottom, original Western blot and original ethidium bromide-stained agarose gels demonstrating the concentration-dependent increase in Cx43 protein and mRNA, respectively, after 24-h stimulation with phenylephrine in relation to GAPDH (con, control; phe, phenylephrine; 10^{-6} M).

phenylephrine-induced increase in Cx43. However, the resulting inhibition of the latter was not complete, and control levels could not be reached (Fig. 2). Furthermore, Western Blot analysis was carried out on cardiomyocytes stimulated with 1 μM phenylephrine (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 phenylephrine. This increase could be blocked by the PKC inhibitor BIM I and by the α_1-adrenozeptor blocker prazosin. In a subsequent experiment, we studied the phosphorylation status of JNK, a protein that is activated downstream of PKC. We observed a significant increase in phosphorylated JNK in relation to total JNK. Again, this increase could be inhibited by BIM I and prazosin (Fig. 3).

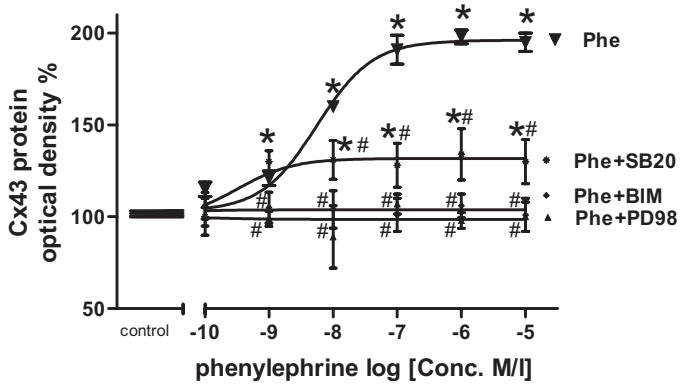

Fig. 2. Inhibition of the phenylephrine-induced up-regulation of Cx43 protein by bisindolylmaleimide I (BIM), SB203580 (SB20), or PD98059 (PD98). Significance versus untreated baseline is indicated by an asterisk ($p < 0.05$). Significance versus phenylephrine is indicated by a # ($p < 0.05$). Please note that bisindolylmaleimide I and PD98059 caused complete inhibition. All data are given as means ± S.E.M. of $n = 6$ experiments.

PCR Results. Thereafter, to determine whether the phenylephrine-induced rise in Cx43 protein might involve regulation on the transcriptional level rather than reduced Cx43 protein degradation, real-time PCR studies were performed. In these experiments, we also found a significant concentration-dependent increase of Cx43 mRNA relative to the housekeeping gene GAPDH with a pEC_{50} of 8.36 ± 0.11 (~4.4 nM), $R^2 = 0.90$ ($n = 10$) (Fig. 1). Because this indicated a possible control of Cx43 expression by α_1-adrenoceptor activation, we tried to elucidate the downstream signaling cascade and possible involvement of p38 and p42/44 MAPK. Therefore, in a second set of experiments, the inhibitors SB203580 or PD98059 or BIM I were administered concomitantly to 1 μM phenylephrine. The PCR analysis of Cx43 mRNA revealed similar findings as described above for the protein level: thus, we found a significant and complete suppression of the phenylephrine-induced increase in Cx43 mRNA by the PKC inhibitor BIM I and by the p42/44 blocker PD98059. However,

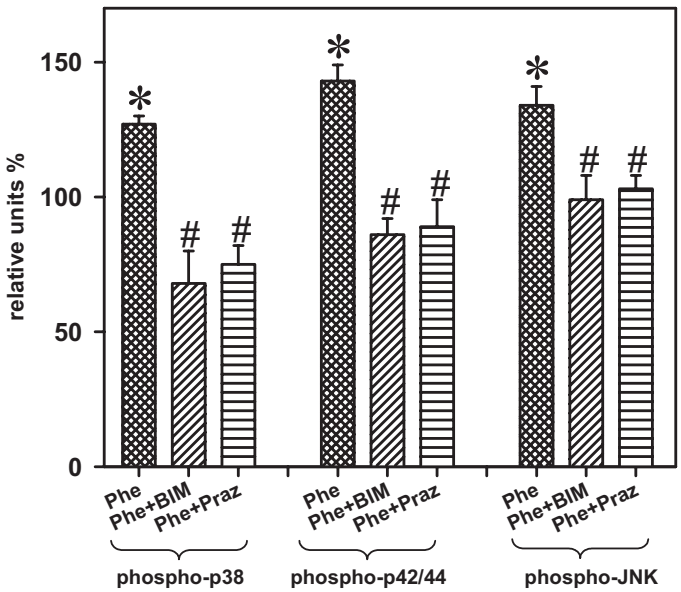

Fig. 3. Expression of the phosphoisoforms of p38, p42/44, or JNK after stimulation with phenylephrine (1 μM) for 24 h in absence or presence of bisindolylmaleimide I (BIM; 5 μM) or prazosin (0.1 μM). Significance versus untreated baseline is indicated by an asterisk ($p < 0.05$). Significance versus phenylephrine is indicated by a # ($p < 0{,}05$). All data are given as means ± S.E.M. of $n = 6$ experiments.

the p38 inhibitor SB203580 showed some different results: on the protein level, SB203580 only partially inhibited the phenylephrine-induced rise in Cx43 protein as described above, whereas on the mRNA level, SB203580 completely inhibited the phenylephrine-induced rise in Cx43 mRNA (Fig. 4).

Analysis of Transcription Factors. The transcription factor c-fos is involved in many intracellular processes, especially in the mitogenic-activated protein cascade signaling (Frödin and Gammeltoft, 1999). Because the above data suggested involvement of p38 and p42/44 MAPKs and JNK, and because these MAPKs regulate c-fos and AP1, we wanted to find out whether the intracellular content of this factor was altered by phenylephrine. For that purpose, Western blot analysis of nuclear and cytosolic extracts of cardiomyocytes stimulated with 1 μM phenylephrine was performed. First, 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 0.095 ± 0.018 (arbitrary units) versus cytosolic fractions 1.93 ± 0.055 (arbitrary units)]. A 24-h stimulation of the cardiomyocytes with phenylephrine resulted in a significant increase in the transcription factor c-fos, in the nuclear fractions, whereas c-fos remained constant at low levels in the cytosolic fractions (Fig. 5, A and B). Furthermore, the AP1, which is a dimer or heterodimer of Fos/Jun, was analyzed in nuclear fractions using the EMSA technique. In these experiments, we could demonstrate that 1 μM phenylephrine, administered for 24 h, significantly up-regulated AP1 content in the nuclear fractions, as could be observed by the shift of the AP1 consensus sequence after administration of the nuclear extracts (Fig. 5C).

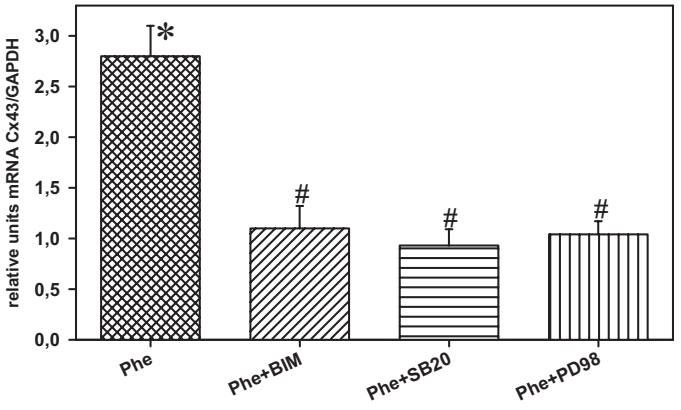

Fig. 4. Induction of Cx43 mRNA stimulation with 1 μM phenylephrine (phe) for 24 h in the absence or presence of bisindolylmaleimide I (BIM), SB203580 (SB20), or PD98059 (PD98). Significance versus untreated baseline is indicated by an asterisk ($p < 0.05$). Significance versus phenylephrine is indicated by a # ($p < 0.05$). All data are given as means ± S.E.M. of $n = 6$ experiments.

Transfection Experiments with Promoter Fragments. Finally, because the above data so far indicated a possible role for AP1 in Cx43 expression, we decided to analyze the effect of phenylephrine on the Cx43 promoter and of deleted mutants of this promoter.

α_1-Adrenoceptor stimulation (1 μM phenylephrine) of cardiomyocytes, previously transfected with the Cx43-promoter EGFP constructs, resulted in a significant increase ($p < 0.05$) in EGFP fluorescence, depending on the length of Cx43-promoter fragments. Phenylephrine stimulation of cardiomyocytes transfected with the smallest Cx43-promoter fragment [91 base pairs (bp)] produced approximately 50% of the fluorescence intensity of the positive control (complete promoter). In the longer promoter fragments, the fluorescence intensity remained nearly constant (approximately 80% of the positive control) up to a promoter length of 2005 bp. The longest promoter fragment (2421 bp) caused the same amount of fluorescence intensity than the complete promoter with 2771 bp. A hyperbolic function could be well fitted to the fluorescence intensity versus promoter fragment-length relationship (Fig. 6) ($R^2 = 0.97$, $p < 0.0001$).

Discussion

In previous experiments, our working group and several other authors have shown that activation of PKC through stimulation of the α-adrenoceptor leads to an enhancement of connexin phosphorylation, assembly, or expression, thereby altering cell communication (Doble et al., 2000; Salameh et al., 2006; Rojas Gomez et al., 2008). Moreover, others could also demonstrate that the MAPK-signaling cascade might play a role in connexin regulation (Inoue et al., 2004; Melcheier et al., 2005). In addition, in good accordance with these data, we found an involvement of MAPK in the regulation of Cx43 expression after stimulation with tumor necrosis factor α (Salameh et al., 2004).

Regarding α_1-adrenoceptor stimulation, our present results demonstrate that a 24-h stimulation of neonatal cardiomyocytes with phenylephrine resulted in a concentration-dependent rise in Cx43 protein as well as in Cx43 mRNA, indicating a regulation of Cx43 on both protein and mRNA level by α_1-adrenoceptor stimulation. The increase in Cx43 could be blocked by either the PKC inhibitor BIM I or inhibitors of the MAPK signaling pathway, demonstrating an involvement of p38 and p42/44. Both proteins as well as the

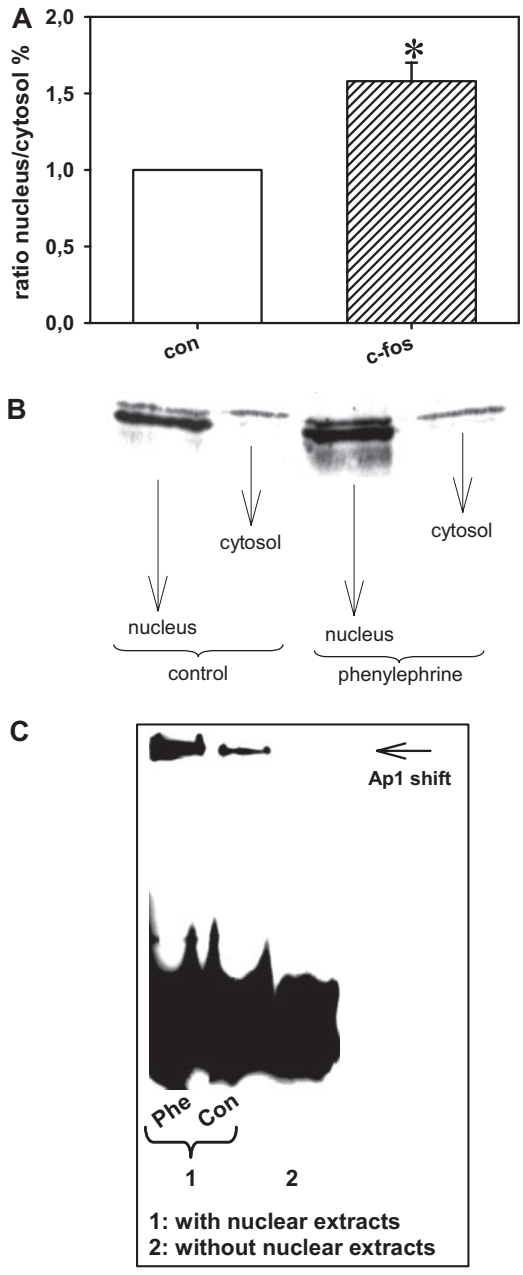

Fig. 5. A, translocation of c-fos from cytosolic to nuclear fraction after stimulation with 1 μM phenylephrine for 24 h. The data are given as means ± S.E.M. from $n = 6$ experiments. Significance versus untreated baseline is indicated by an asterisk ($p < 0.05$). B, original example of Western blot showing nuclear and cytosolic c-fos presence for untreated baseline conditions and after stimulation with 1 μM phenylephrine for 24 h. C, original AP1 EMSA from untreated control cells and from cells after stimulation with 1 μM phenylephrine for 24 h. Please note the positive AP1 shift in both nuclear extract preparations (1), which was more pronounced after phenylephrine, and the negative AP1 shift in the absence of nuclear extracts (2) (con, control; phe, phenylephrine).

third protein involved in the MAPK-signaling cascade, JNK, were further analyzed for their phosphorylation status. It could be clearly demonstrated that after phenylephrine stimulation, p38 as well as p42/44 and JNK showed an increase in their phosphorylated forms, suggesting an increase in activity of these kinases. The rise in the phosphorylated forms of all three kinases was inhibited by both BIM I or prazosin, exhibiting a dependence on the PKC and α_1-adrenoceptor. Because it is well known that the Cx43 promoter contains an

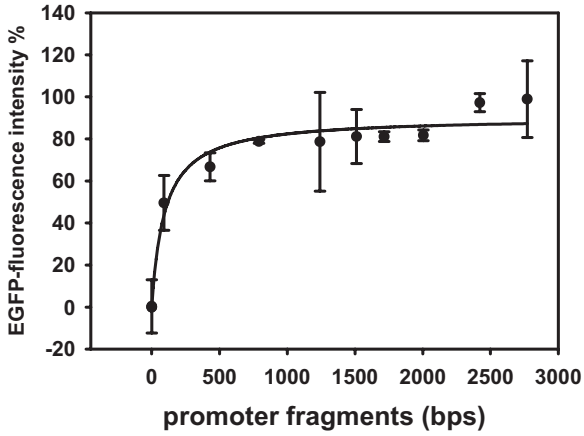

Fig. 6. The graph depicts the relative green fluorescence intensity of neonatal rat cardiomyocytes transfected with the Cx43-promoter EGFP construct and its fragments. The x-axis gives the length of the promoter fragment, and the y-axis shows the EGFP intensity. A hyperbolic function of the form $y = a^*x/(b + x)$ could be adapted and revealed a 50% effect promoter length of 94 ± 31 bp ($R^2 = 0.98$).

AP1 binding site (Geimonen et al., 1996), the influence of phenylephrine on AP1 was examined using the EMSA technique. In cardiomyocytes stimulated with the α_1-adrenoceptor agonist, a shift of the AP1 consensus sequence could be detected after administration of stimulated nuclear extracts, in contrast to the cytosolic extracts that caused no shift, implying that AP1 is indeed involved in the phenylephrine-induced rise in Cx43. Thus, α_1-adrenoceptor stimulation seems to activate PKC, p38, p42/44, and JNK, resulting in c-fos translocation to the nucleus.

Finally, to investigate the importance of AP1 in the phenylephrine-dependent regulation of Cx43 transcription, a vector containing the EGFP under control of the Cx43 promoter was constructed, and the promoter was truncated into fragments of distinct length starting from TIS and extending upstream. The smallest fragment (91 bp) produced approximately 50% of the fluorescence intensity of the complete promoter. Because an AP1 site has been identified after TIS at nucleotides 38 to 44 (De Leon et al., 1994; Geimonen et al., 1996), this may indicate that the AP1 site of the Cx43 promoter is located near the TIS, and that phenylephrine presumably causes an up-regulation of AP1 that subsequently binds to this site and initiates Cx43 transcription. This theory fits our above data, which showed α_1-adrenoceptor-dependent activation of the PKC p38, p42/44, and JNK pathway with nuclear c-fos translocation.

However, it is necessary to consider some incongruity in our data regarding the effect of SB203580. We found the same degree of inhibition of phenylephrine-induced Cx43 mRNA with SB203580 as with PD98059 or with BIM, whereas regarding Cx43 protein, SB203580 resulted in only partial (but significant) inhibition, in contrast to PD98059 and BIM, which completely inhibited phenylephrine-induced up-regulation of Cx43 protein. This might indicate that p38 stimulation serves as only a cofactor in Cx43 regulation, whereas p42/44 activation seems to be essential. On the other hand, this data might also be interpreted as a hint of the changes in mRNA half-life time, stability, or of the changed translational control.

Our investigation using a specific α_1-adrenoceptor agonist (i.e., phenylephrine) revealed that phenylephrine leads to an

up-regulation of Cx43 protein and mRNA levels, both with an EC_{50} of ~5 nM, which supports the view of a specific action on α_1-adrenoceptors (Bylund et al., 1994).

Previous studies have shown that the α_1-adrenoceptor is involved in hypertrophic responses in cardiomyocytes, acting via G_q-PKC signal transduction cascade (Schlüter and Piper, 1992; Schlüter et al., 1999; Pönicke et al., 2002). Moreover, in uterine muscle, it was shown that Cx43 can be induced by stimulation of PKC (Geimonen et al., 1996). In agreement with these results, our findings show that the phenylephrine-induced rise in Cx43 expression is suppressed by BIM I. It is known that the α_1-adrenoceptor is coupled to PKC via a $G_{q/11}$ protein, and, of interest, it was previously shown by other working groups that other $G_{q/11}$-coupled receptors, namely the AT_1 and ET_1 receptors, also induce Cx43 expression (Dodge et al., 1998; Polontchouk et al., 2002). Furthermore, Polontchouk et al. (2002) demonstrated that the signaling pathway via the AT_1 receptor involves an activation of p38 and p42/44 protein kinases. They also found a dependence of Cx43 expression after stimulation with angiotensin on p38 and p42/44 MAPK, because the angiotensin signal was abolished after administration of the MAPK blockers SB203580 or PD98059. Moreover, stimulation of α_1-adrenoceptors not only comprises the phospholipase C/PKC pathway, but it also implicates signaling via the family of small G-proteins such as Ras and Rho (Ramirez et al., 1997; Barki-Harrington et al., 2004). Both of them are known to stimulate JNK and mitogen-activated protein kinase kinase, thereby transducing an extracellular signal to the nucleus. Accordingly, Schlüter et al. (1999) found an activation of extracellular signal-regulated kinase (ERK) (= p42/44 MAPK) after administration of phenylephrine, and Rohde et al. (2000) reported that stimulation of the α_1-adrenoceptor resulted in an activation of not only ERK but also of the p38 MAPK. These working groups demonstrated that activation of the MAPK-signaling cascade (i.e., the JNK, ERK, and p38 pathway) causes an enhanced protein synthesis and contributes to hypertrophic responses of cardiomyocytes. All of these findings suggest that stimulation of the α_1-adrenoceptor results in an increase in cardiac protein synthesis, cell growth, and Cx43 gap junction protein. In clinical findings, Kostin et al. (2004) showed that in patients with compensated left ventricular hypertrophy due to aortic stenosis, the amount of Cx43 in the heart was elevated, which would fit to our data, because in that clinical situation, catecholamine release is typically enhanced.

At this point, we need to consider the experimental conditions with regard to the duration of α_1-adrenoceptor stimulation: Cx43, although being an integral membrane protein, exhibits a high turnover rate with a half-life time of only 1.5 h (Beardslee et al., 1998). Thus, within 24 h, we can expect a severalfold turnover of Cx43. In this regard, the 24-h stimulation period used in the present study may be considered to simulate subchronic stimulation.

Moreover, the question arises as to whether the α_1-adrenoceptor stimulation-dependent Cx43 up-regulation seen here in cultured cells may be transferred to the in vivo situation in the adult organism. Using 24-h phenylephrine infusion in conscious adult rats, we could show a similar nearly 2-fold Cx43 up-regulation under in vivo conditions (Salameh et al., 2006).

The last point to consider is the role of AP1 in the regulation of Cx43 gene expression by phenylephrine. There are several reports of an AP1 binding site in the Cx43 promoter near the TIS (De Leon et al., 1994; Geimonen et al., 1996; Mitchell and Lye 2005), and, as shown in our results and also by other working groups (Hines and Thorburn, 1998; Spector et al., 2000), stimulation of the α_1-adrenoceptor leads to an up-regulation of c-fos and c-jun, which then cause formation of the AP1 protein, thereby regulating Cx43 gene transcription. Our finding with the truncated Cx43 promoter that a minimum of 91 bp was sufficient to obtain 50% of the EGFP fluorescence of the complete promoter suggests that an AP1 binding site near the TIS must be existent. Indeed, such a site was also described by Teunissen and coworkers (Teunissen et al., 2003; Teunissen and Bierhuizen 2004) who could show that, among other transcription factor bindings sites (i.e., SP1 and SP2), an AP1 site near TIS exists. The importance of this first AP1 site in regulating Cx43 gene transcription was also pointed out by Geimonen et al. (1998) and Lefebvre et al. (1995). Echetebu et al. (1999) showed that for optimal Cx43-promoter activity, both transcription factors, i.e., AP1 and SP1, are necessary, but unless the AP1 and SP1 are ubiquitous transcription factors involved in many cellular processes, other factors regulating Cx43 gene expression must exist.

Taken together, the data in our present study show that phenylephrine-induced Cx43 up-regulation is transduced via PKC, p38, p42/44, JNK activation, and c-fos nuclear translocation, and it seems to be predominantly mediated via induction of binding of AP1 to a recognition site within the first 100 bp near the TIS of the Cx43 promoter. Because similar signal transduction pathways were identified for ET_A and AT_1 receptor stimulation (Polontchouk et al., 2002), this, moreover, might be a general pathway to regulate Cx43 via $G_{q/11}$-coupled receptors.

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