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Differential Regulation of Gap Junction Proteins Connexin 40 and Connexin 43 in Cardiomyocytes

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Abstract: The goal of this study was to find out, whether Cx43 and Cx40 expression may be chronically regulated via adenylyl cyclase (AC)/cAMP/protein kinase A (PKA) and protein kinase C (PKC) pathways. Therefore confluent cultures of neonatal rat cardiomyocytes were incubated 24 h with various concentrations of either dibutyryl-cAMP (10-1000 μM), forskolin (0,1-10 μM) or the protein kinase C (PKC) activator phorbol-12,13-didecanoate (PDD) (1 nM-10 μM) in absence or in presence of the p38-MAP-kinase inhibitor SB203580 (10^{-5} M) or the MEK1-inhibitor PD98059 (10^{-5} M). Connexin 40 and 43 expression was investigated by Western blotting and PCR. Incubation with either db-cAMP, forskolin or PDD led to a dose-dependent significant ($p<0.05$) increase in Cx43 expression as revealed by Western blot. These results were also confirmed by PCR studies indicating de-novo synthesis of Cx43. In contrast Cx40 expression was not altered by activation of the PKA pathway. However the PKC activator PDD enhanced both connexins Cx43 and Cx40 on the protein level. In additional PCR experiments only the PDD-induced Cx43 increase was associated with an increase in Cx43-mRNA, whereas the Cx40-mRNA remained unchanged. The increases in Cx43 protein content could be completely suppressed by SB203580 ($p<0.05$) but not by PD98059. In absence of a stimulating drug, these inhibitors (SB203580 or PD98059) did not affect Cx43 content. In PCR studies the increase in Cx43-mRNA under the influence of db-cAMP, forskolin or PDD could also be completely suppressed by SB203580. From these results it can be concluded, that (a) Cx43 and Cx40 are differentially regulated and (b) the Cx43 expression can be regulated via AC/cAMP/PKA and PKC dependent pathways, (c) from the PCR results that the up-regulation in Cx43 seems to be attributable to enhanced de-novo synthesis and that (d) the activation of p38 MAP kinase is a common pathway for regulation of Cx43 expression in rat cardiomyocytes.

Key words: Gap junction, connexins, protein kinase A, cAMP, protein kinase C, MAP-kinase

INTRODUCTION

Gap junctional channels provide intercellular communication thereby forming a functional syncycium. These channels allow electrical and metabolic coupling between neighbouring cells and are important for a regular function of all organs. One gap junction channel is composed of two hexameric hemichannels of each neighbouring cell and the hemichannels themselves consist of certain proteins the connexins. In mammalian hearts several connexins are expressed: connexin 40 (Cx40), connexin 43 (Cx43), connexin 45 (Cx45)[1]. Among these Cx43 is especially found in cardiomyocytes of the ventricle whereas Cx40 is predominantly localised in the atria and in small amounts in the conduction system[2]. Connexin 45 is expressed in embryonic stages of the heart and in very small amounts also in the Purkinje system of adult hearts. The gap junction channels formed by different connexins differ in their biophysical properties i.e. regulation, permeability and conductivity.

While much work has been carried out on the role of protein kinases in acute regulation of intracellular communication by connexin phosphorylation[3-9], only little is known on the effects of chronic stimulation of protein kinases on gap junction protein expression[10, 11]. On the other hand Dodge *et al.*[12] showed that 24 h stimulation of cardiomyocytes with angiotensin increased gap junction expression. Moreover, Polontchouk *et al.*[13] demonstrated that 24 h treatment with either angiotensin

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II or endothelin-1 resulted in enhanced Cx43 but not Cx40 expression in neonatal rat cardiomyocytes and that the increased Cx43 content led to enhanced intercellular electrical coupling.

However, a more systematic and comparative investigation of the pathways regulating connexin 43 expression is still lacking. Thus, the aim of the present study was to investigate the effect of chronic stimulation of two typical and important signal transduction pathways on Cx43 and Cx40 expression: the adenylyl cyclase (AC)/cAMP/protein kinase A (PKA) pathway and the protein kinase C (PKC) pathway. Moreover the role of mitogenic activated protein kinases (MAP-kinases) were investigated, since several authors reported interactions between MAP-kinases and PKA and PKC pathways[14-16].

MATERIALS AND METHODS

db-cAMP, forskolin, PDD and SB203580 were obtained from Alexis Biochemicals (San Diego, CA). MEK1 inhibitor PD98059 was purchased from Cell Signaling (New England Biolabs). Polyclonal rabbit anti-Cx40 and monoclonal mouse anti-Cx43 were bought from Chemicon (Temecula, USA). Secondary antibody horseradish-labeled antibody, M199 and all other chemicals used were obtained from Sigma (Deisenhofen, Germany). Foetal calf serum and collagenase II were purchased from Gibco Life Technologies (Karlsruhe, Germany). The primers were synthesised by BioTeZ GmbH Berlin, Germany.

Cell culture: Cardiomyocytes were isolated and cultured according the following protocol[13]. Ventricles of new-born Wistar rats were digested in collagenase II solution, centrifuged and after a pre-plating period to remove non cardiac cells resuspended in M199 medium containing 2 mM L-glutamine, 100 mg mL^{-1} streptomycin and penicillin, 1% foetal calf serum and 10% horse serum (to inhibit fibroblast growth). The cells were seeded in Petri dishes coated with 0.1% gelatine and medium was changed three times a week. After reaching confluence the cells were exposed to different concentrations of db-cAMP, forskolin, PDD with or without MAP-kinase inhibitors for 24 h and subsequently were analysed using immunoblotting or PCR. db-cAMP was used in concentrations between 10-1000 μM, forskolin in concentrations between 0.1-10 μM and the protein kinase C (PKC) activator phorbol-12,13-didecanoate (PDD) in concentrations between 1 nM-10 μM. In a second series of experiments the agonists were administered as before but in presence of the p38-MAP-kinase the inhibitor SB203580(4-(4-fluorophenyl)-2-(4-methyl-sulfinylphenyl)-5-(4-pyridyl)-1H-imidazole) or the MEK 1 (extracellular regulated kinase kinase) the inhibitor PD98059 (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one) were both used at a concentration of 10 μM. PD98059 and SB203580 are specific inhibitors for MEK1 or p38 MAP-kinase, respectively[17-19].

After 24 h treatment protein extracts were generated from cardiomyocytes using extraction buffer containing Triton-X100 and protease inhibitors (K_2HPO_4 20 mM pH=7,9, EDTA 1 mM, aprotinin 10 μg mL^{-1}, leupeptin 0.5 mg mL^{-1}, pepstatin A 7 μg mL^{-1}, Triton-X100 1%, PMSF 1 mM). Total protein concentration was determined in the supernatants according to the method described[20]. Thereafter Western Blot analysis of the Cx40 and Cx43 content were carried out.

In further experiments for investigation of Cx43 and Cx40-mRNA confluent monolayers of cardiomyocytes were either stimulated with db-cAMP (100 μM), forskolin 10 μM or PDD 1 μM with or without additional treatment of 10^{-5} M SB203580 for 24 h. For RNA isolation cells were harvested using TRIZOL (Gibco, Germany) and cDNA was synthesised by reverse transcription using 1 μg total RNA. PCR was carried out with primer pairs flanking CX43, Cx40 or the housekeeping-gene GAPDH. The products of expected size (Cx43 600 bp, Cx40 216 bp and GAPDH 415 bp) were visualised in ethidiumbromide stained 1.5% agarose gels.

Western blots: The cell lysates were mixed with gel loading buffer according to Laemmli following classical protocols[21] and for electrophoresis 30 μg protein per slot of each protein sample was fractionated through a 4% stacking and 10% running SDS-polyacrylamide gel.

Proteins where then transferred electrically (semi dry blot) on to a nitrocellulose membrane and blocked with 6% low fat milk blocker at 4°C overnight. Primary antibody to Cx40 or Cx43 diluted 1:500 were applied for 2 h at room temperature. Thereafter, the blots were washed three times with phosphate buffered saline (PBS: containing NaCl 137 mM, KCl 2.68 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.47 mM and 0.1% Tween-20) and were incubated with secondary horse-radish peroxidase labeled antibody diluted 1:1000 for 1 h at room temperature. Thereafter, connexins were detected using the iodophenol/luminol system by application of the ECL (enhanced chemiluminescence) western blot detection kit from Amersham Pharmacia Biotech. Immunoblots were incubated in iodophenol/luminol reaction mixture (60s) and exposed to X-ray film to detect chemiluminescence. The connexin bands were imaged on a scanner and the pictures were digitised and analysed with BioRad software (BioRad, München, Germany). After background

subtraction grey scale values of connexin signals in experimental preparations were compared with signals of the untreated control cells, which were normalized to a value of 1.0[22].

Reverse transcription and PCR amplification: Confluent cell monolayers of neonatal rat cardiomyocytes were harvested and RNA was isolated using TRIZOL (Gibco BRL, Germany). Thereafter RNA was reverse transcribed from 1 μg total RNA with random hexamer to generate fist-strand cDNA using standard protocols[23]. After first-strand cDNA was prepared 1 μl cDNA was put together with PCR reagents to make a 25 μl solution containing 1 U Taq DNA polymerase (Gibco, Germany), 10x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP and 0.5 μl of each primer. The amplification was carried out using the following primer according[24]: Cx43 antisense 5′-TTG TTT CTG TCA CCA GTA AC-3′, sense 5′-GAT GAG GAA GGA AGA GAA GC-3′, Cx40 antisense 5′-TCC CGT TCA CCT CTT TCC AG-3′, sense 5′-CCT TCC CCA TCT CCC ACA TT-3′,GAPDH antisense 5′-CCG CCT GCT TCA CCA CCT TCT-3′, sense 5′-GTC ATC ATC TCC GCC CCT TCC-3′. The cDNA amplification products of Cx43 were predicted to be 600 bp, of Cx40 216 bp and of GAPDH 415 bp, respectively. The products were visualised and analysed in ethidiumbromide stained 1.5% agarose gels. Connexin expression was normalized to GAPDH expression.

Statistical analysis: The concentration-response curves have been analysed for C_{max}, EC_{50} and Hill slope and were fitted to a sigmoidal curve using GraphPadPrism software (GraphPad Software, San Diego, CA, USA). A two-factorial analysis of variance (ANOVA) was performed. If ANOVA indicated significant differences the data were additionally analysed with the Tukey HSD test. For statistical analysis "Systat for Windows, ver. 5.02" (Systat Inc., Evanston, USA) software was used.

RESULTS

Western blot analysis

Stimulation of AC/cAMP/PKA pathway: To determine whether stimulation of the PKA pathway can modify connexin expression, neonatal cardiomyocytes were stimulated with increasing concentrations of db-cAMP (10^{-5} to 10^{-3}M) for 24 h and expression of Cx40 and Cx43 was measured by immunoblotting. As shown in Table 1, Fig. 1a and b db-cAMP caused a significant ($p<0.05$) increase in total Cx43 amount in a dose-dependent and saturable manner. The EC_{50} value calculated from the dose-response curve was $3*10^{-4}$ M. The Hill-slope of the concentration-response curve of the Cx43 expression was 1.0. Stimulation of the cardiomyocytes with increasing concentrations of the adenylyl cyclase activator forskolin

Table 1: Characteristics of the concentration-response-curve for the expression of cardiac connexins

Treatment	log EC_{50}	R^2
Western blot Cx43		
db-cAMP	-3.5±0.23	0.98
Forskolin	-8.2±0.12	0.99
PDD	-7.8±0.14	0.98
Western blot Cx40		
PDD without SB203580	-7.9±0.20	0.97
PDD with SB203580	-8.1±0.20	0.98

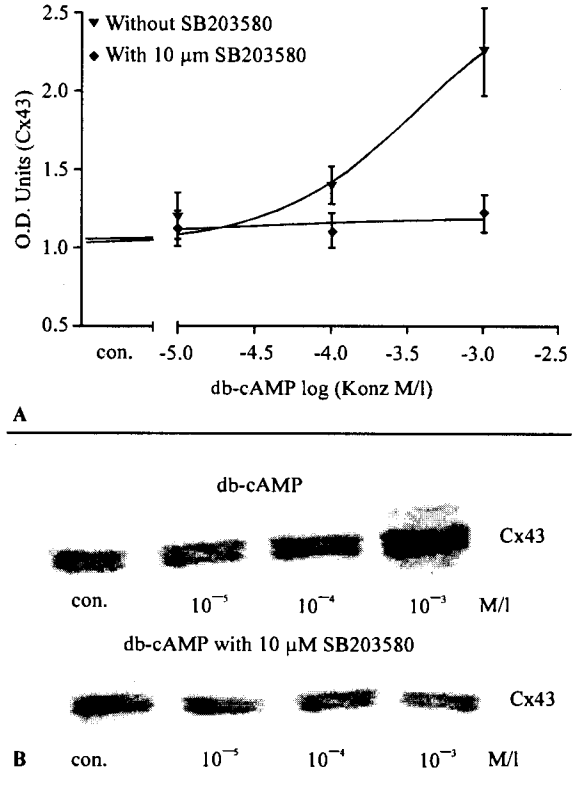

Fig. 1A: Western blot results for Cx43 in neonatal cardiomyocytes exposed to db-cAMP (10^{-3} to 10^{-5} M/l) for 24 h in absence or presence of the p38 MAP kinase inhibitor SB203580 (10 μM). All values are given as means±SEM of n=4 experiments in optical density (OD) units relative to the untreated control cells (control=1)

B: Original Western blot for Cx43 in neonatal cardiomyocytes exposed to db-cAMP (10^{-3} to 10^{-5} M/l) for 24 h in absence or presence of the p38 MAP kinase inhibitor SB203580 (10 μM). con=control

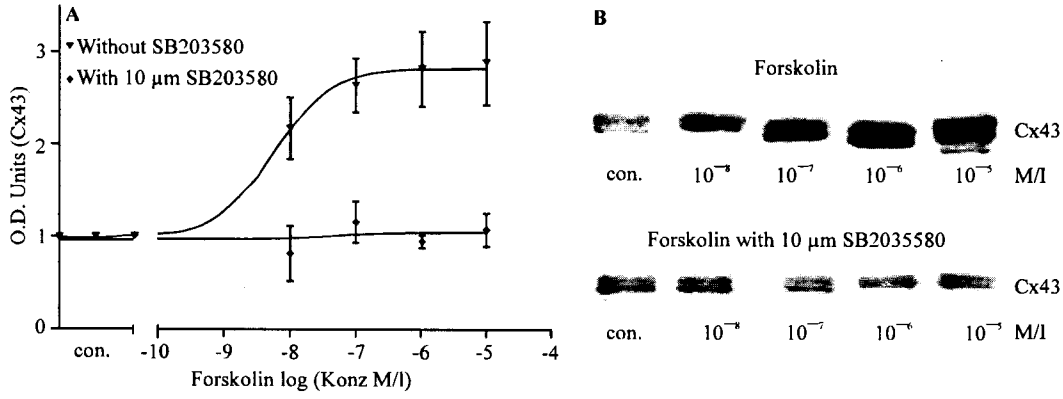

Fig. 2A: Western blot results for Cx43 in neonatal cardiomyocytes exposed to forskolin (10^{-5} to 10^{-8} M/l) for 24 h in absence or presence of the p38 MAP kinase inhibitor SB203580 (10 µM). All values are given as means±SEM of n=4 experiments in optical density (OD) units relative to the untreated control cells (control=1)

B: Original Western blot for Cx43 in neonatal cardiomyocytes exposed to forskolin (10^{-5} to 10^{-8} M/l) for 24 h in absence or presence of the p38 MAP kinase inhibitor SB203580 (10 µM). con=control

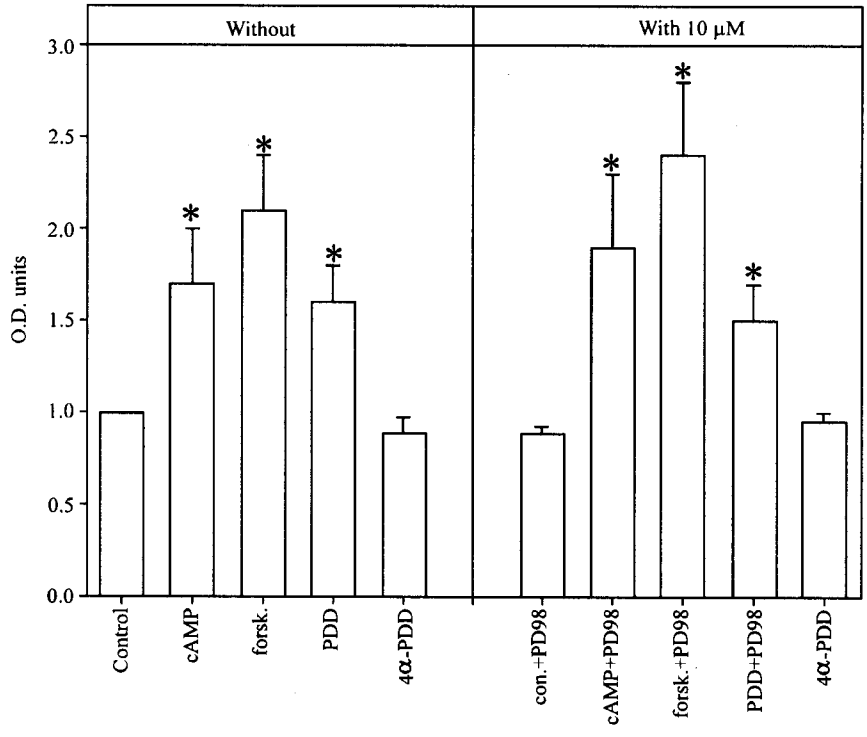

Fig. 3: Western blot results for Cx43 in neonatal cardiomyocytes exposed to either, 10^{-7} M phorbol-12,13-didecanoate (PDD), or its inactive form 4α-PDD, 10^{-5} M forskolin (10^{-7} M) or 10^{-4} M db-cAMP (dibutyryl-cAMP) for 24 h in absence or presence of the MEK inhibitor PD98059 (10 µM). All values are given as means±SEM of n=4 experiments in optical density (OD) units relative to the untreated control cells (control=1). Significant differences versus control are indicated by an asterisk ($p<0.05$). There were no significant differences between cells in absence and presence of PD98059 ($p<0.05$)

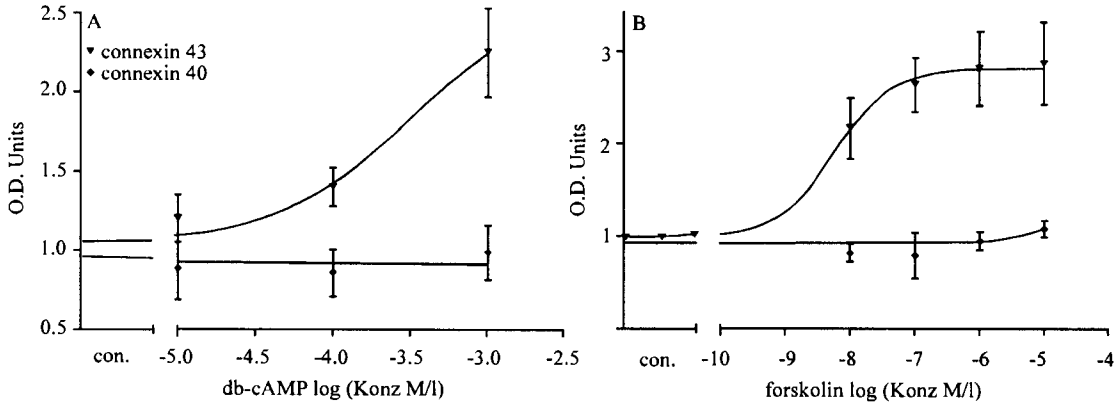

Fig. 4A: Western blot results for Cx43 and Cx40 in neonatal cardiomyocytes exposed to db-cAMP (10^{-3} to 10^{-5} M/l) for 24 h. All values are given as means±SEM of n=4 experiments in optical density (OD) units relative to the untreated control cells (control=1)

B: Western blot results for Cx43 and Cx40 in neonatal cardiomyocytes exposed to forskolin (10^{-5} to 10^{-8} M/l) for 24 h All values are given as means±SEM of n=4 experiments in optical density (OD) units relative to the untreated control cells (control=1)

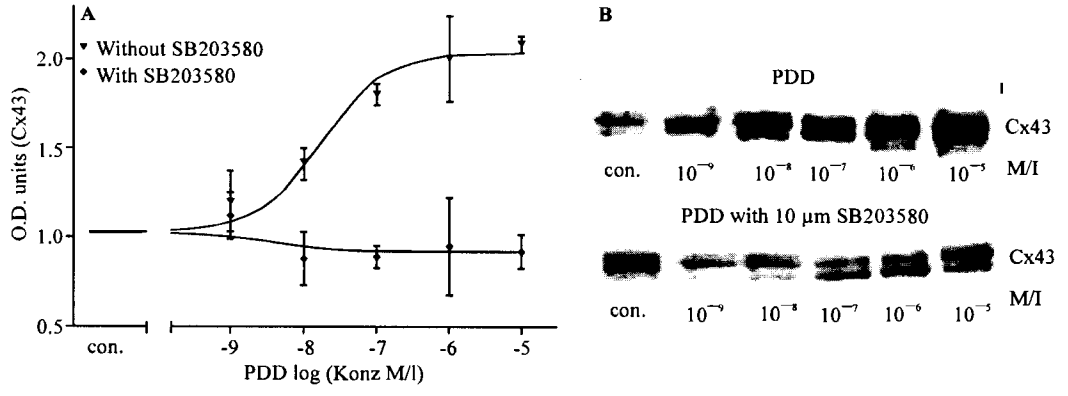

Fig. 5A: Western blot results for Cx43 in neonatal cardiomyocytes exposed to PDD (phorbol-12,13-didecanoate) (10^{-5} to 10^{-9}M) for 24 h in absence or presence of the p38 MAP kinase inhibitor SB203580 (10 µM). All values are given as means±SEM of n=4 experiments in optical density (OD) units relative to the untreated control cells (control=1)

B: Original Western blot for Cx43 in neonatal cardiomyocytes exposed to PDD (10^{-5} to 10^{-9}M) for 24 h in absence or presence of the p38 MAP kinase inhibitor SB203580 (10 µM). con=control

(0.01, 0.1, 1 and 10 µM) also resulted in a significant and dose-dependent increase of Cx43 expression with an EC_{50} value of $6.7*10^{-9}$M, Hill slope=1 (Table 1, Fig. 2a and b). Representative western blots as a compilation of the experiments are shown in the lower panels of Fig. 1 and 2.

In a second series of experiments the p38-MAP-kinase inhibitor SB203580 (10 µM) was applied simultaneously with either db-cAMP or forskolin. The additional application of the p38-MAP-kinase inhibitor resulted in a significant ($p<0.05$) and complete suppression of the db-cAMP or forskolin induced effect on Cx43 expression (Fig. 1 and 2).

In subsequent experiments a possible additional effect of MEK1 pathway was elucidated. Therefore, a single dose of 10^{-3} M db-cAMP or 10^{-5} M forskolin was applied to the cardiomoycytes and another MAP-kinase inhibitor the specific MEK1-inhibitor PD98059 was administered concomitantly at a concentration of 10 µM. In contrast to the p38 MAP-kinase inhibitor, the MEK1-inhibitor did not influence the increase in Cx43 content induced by PKA stimulation (Fig. 3).

In contrast to Cx43 the Cx40 expression could not be stimulated neither by db-cAMP nor by forskolin (Fig. 4). Cx40, which gives only a weak signal, is not up-regulated

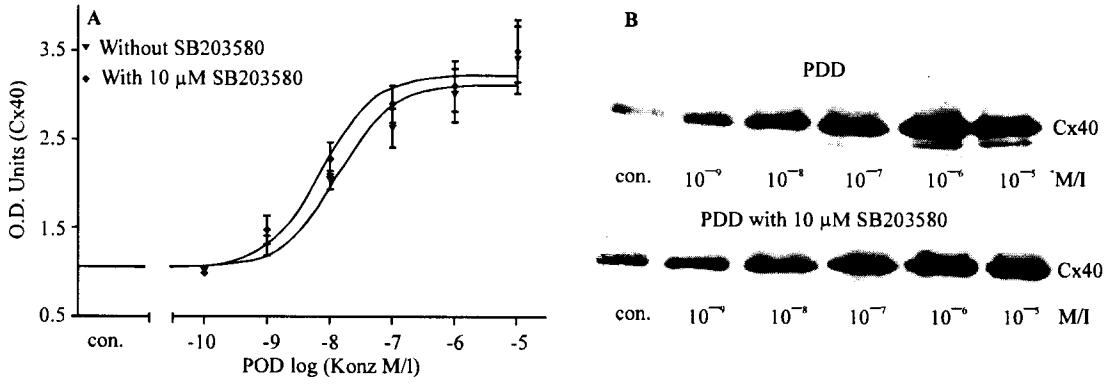

Fig. 6A: Western blot results for Cx40 in neonatal cardiomyocytes exposed to PDD (phorbol-12,13-didecanoate) (10^{-5} to 10^{-9}M) for 24 h in absence or presence of the p38 MAP kinase inhibitor SB203580 (10 µM). All values are given as means±SEM of n=4 experiments in optical density (OD) units relative to the untreated control cells (control=1)

B: Original Western blot for Cx40 in neonatal cardiomyocytes exposed to PDD (10^{-5} to 10^{-9}M) for 24 h in absence or presence of the p38 MAP kinase inhibitor SB203580 (10 µM) con=control

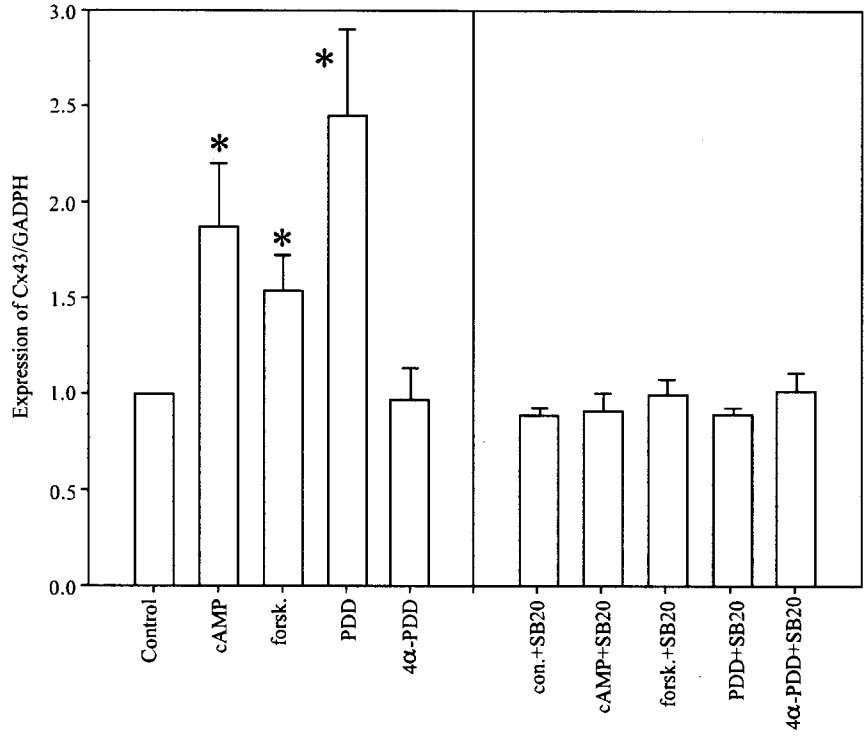

Fig. 7A: PCR results for Cx43-mRNA in neonatal cardiomyocytes exposed to either 10^{-7} M phorbol-12,13-didecanoate (PDD), or the inactive phorbolester 4α-PDD, 10^{-5} M forskolin or 10^{-4} M db-cAMP (dibutyryl-cAMP) for 24 h in absence or presence of the p38 MAP kinase inhibitor SB203580. All values are given as means±SEM of n=4 experiments as relative expression of Cx43-mRNA related to the housekeeping GAPDH-mRNA. Significant differences versus control (control=1) are indicated by an asterisk ($p<0.05$)

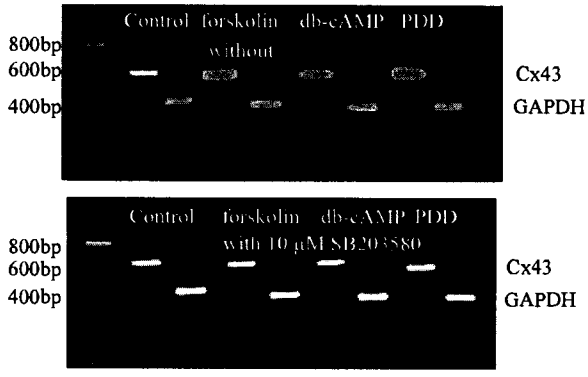

Fig. 7B: Representative PCR analysis of Cx43 and GAPDH mRNA with 24 h treatment with either 10^{-7} M phorbol-12, 13-didecanoate (PDD), 10^{-4} M db-cAMP (dibutyryl-cAMP) or 10^{-5} M forskolin. The cDNA amplification products of Cx43 were predicted to be 600 bp, of GAPDH 415 bp, respectively

by stimulation of the PKA pathway and stays at control levels.

Stimulation of PKC pathway: To elucidate the influence of the PKC the phorbolester PDD was applied in concentration between 1 nM 10 µM and the Cx40 and Cx43 content was measured by immunoblotting. As shown in Fig. 5 PDD increases Cx43 content significantly ($p<0.05$) in a dose-dependent and saturable manner. The EC_{50} value calculated from the dose-response curve was $1.8x10^{-8}$ M with a Hill-slope of 1.0 (Table 1). Again, the increase in Cx43 by PDD could be completely and significantly inhibited by simultaneous application of the p38-MAP-kinase inhibitor SB203580 (10 µM) ($p<0.05$) (Fig. 5), whereas the MEK1-inhibitor PD98059 10^{-5} M did not affect the PDD-induced increase in Cx43 content (Fig. 3)

In contrast to the activation of the PKA pathway by db-cAMP or forskolin, activation of the PKC by PDD resulted in a significant and dose dependent increase in Cx40 protein content ($p<0.05$) with an EC_{50} of $1.3x10^{-8}$ M (Table 1). This increase could not be inhibited by the p38 MAP-kinase inhibitor SB203580 (10 µM) (Fig. 6).

PCR analysis: The PCR analysis of Cx43 mRNA revealed similar findings as described above on the protein level. Thus, a significant increase in Cx43 mRNA relative to the housekeeping gene GAPDH mRNA was found under the influence of a single dose of either db-cAMP (10^{-3} M) or forskolin (10^{-5} M) or PDD (10^{-6} M) (Fig. 7a and b). The expression of GAPDH mRNA was not altered by the different treatments. The increases in Cx43-mRNA could be completely suppressed by additional treatment with SB203580 (10 µM) (Fig. 7a), indicating transcriptional regulation of Cx43 expression via p38 MAP-kinase.

In accordance to the protein data (see above) Cx40 mRNA could neither be stimulated by db-cAMP nor by forskolin. However, while PDD enhanced Cx40 protein content, Cx40-mRNA was not increased after PDD application (Fig. 8a and b).

DISCUSSION

In previous investigations several authors have shown, that acute activation of the adenylyl cyclase/cAMP/PKA pathway or activation of the PKC can regulate connexin phosphorylation and expression thereby altering cell coupling and communication[25-28]. However, little is known on the effects of chronic stimulation of intracellular signaling cascades such as adenylyl cylase, PKC and the role of p38 and p42/44-MAP kinases.

In general, the results of this study demonstrate that the expression of the gap junction protein connexin 43 is not constant but can be regulated. We found an up regulation of Cx43 by stimulation of the adenylyl cyclase/cAMP/PKA and the PKC pathway. This finding of a regulation of Cx43 content would allow a cell to adapt its communication to changing conditions of the neighbouring cells. It is known that in certain physiological and pathophysiological conditions connexin content can be altered: thus, in early stages of renovascular hypertension enhanced Cx43 content has been found in guinea pig hearts[29]. In contrast, in patients suffering from ischemic cardiomyopathy a reduction in Cx43 content has been found[30]. In hypertensive rats Bastide *et al.*[31] found reduced Cx43 but enhanced Cx40 expression. However, the pathways involved in this chronic regulation of Cx43 and Cx40 expression are only partially understood.

Stimulation of the AC/cAMP/PKA pathway by either db-cAMP or by stimulation of the adenylyl cyclase using forskolin resulted in an increase in Cx43 content in good accordance to the findings of Darrow *et al.*[10,11] who showed that 24 h db-cAMP resulted in increased Cx43 expression in neonatal rat cardiomyocytes. Interestingly, in the present study this was blocked by the pyridinyl imidazole SB203580, a substance binding to the ATP binding pocket of p38 MAP-kinase thereby inhibiting p38 MAP-kinase activity[19]. The specificy of SB203580 10 µM for inhibition of p38 MAP-kinase has been established in cardiomyocytes previously by others[32]. The effect of SB203580 in this study indicated, that stimulation of

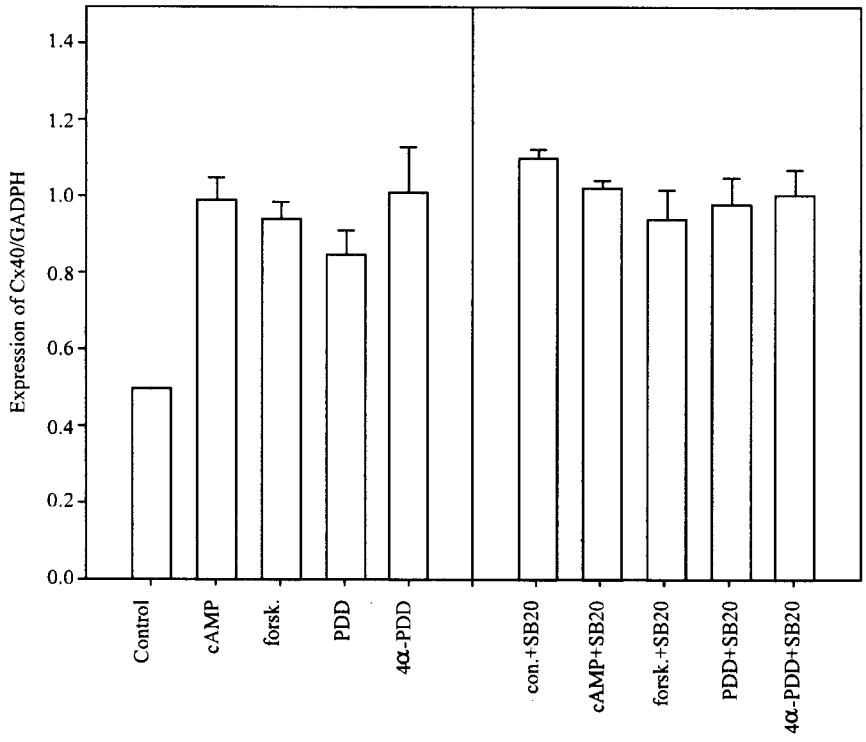

Fig. 8A: PCR results for Cx40-mRNA in neonatal cardiomyocytes exposed to either 10^{-7} M phorbol-12,13-didecanoate (PDD), or the inactive phorbolester 4α-PDD, 10^{-5} M forskolin or 10^{-4} M db-cAMP (dibutyryl-cAMP) for 24 h in absence or presence of the p38 MAP kinase inhibitor SB203580. All values are given as means±SEM of n=4 experiments as relative expression of Cx40-mRNA related to the housekeeping GAPDH-mRNA. Significant differences versus control (control=1) are indicated by an asterisk ($p<0.05$)

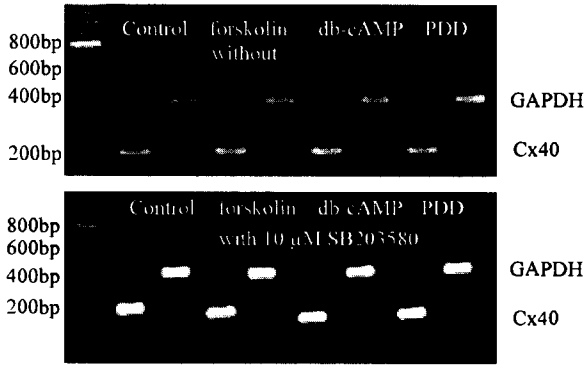

Fig. 8B: Representative PCR analysis of Cx40 and GAPDH-mRNA with 24 h treatment with either 10^{-7} M phorbol-12,13-didecanoate (PDD), 10^{-4} M db-cAMP (dibutyryl-cAMP) or 10^{-5} M forskolin. The cDNA amplification products of Cx40 were predicted to be 216 bp, of GAPDH 415 bp, respectively

adenylyl cyclase with forskolin acting via cAMP and possibly via PKA activates p38 MAP-kinase, while MEK-1 does not seem to be involved, because the MEK-1 inhibitor PD98059 did not exhibit an effect. It is noteworthy that the use of 10 μM PD98059 for specific inhibition of MEK-1 has also been established[28]. The hypothesis that forskolin and db-cAMP may act finally via PKA is further supported by the finding that PKA can activate p38 MAP-kinase in adult mouse cardiomyocytes[33]. It has been suggested by these authors and by the work of Saxena and colleagues[34,35] that activation of PKA may inhibit phosphatase 1 (PP1) via phosphorylation of its regulatory subunit and/or may phosphorylate protein tyrosine phosphatases resulting in an inhibition of their interaction with p38 MAP-kinase, thereby enhancing p38 MAP-kinase activity. Thus, our data indicate that stimulation of the AC/cAMP/PKA cascade leads to enhanced Cx43 content via p38 MAP-kinase activation.

Secondly, this study that stimulation of PKC via the phorbolester PDD led to increased Cx43 content. The

effect seemed to be specific since the inactive analogue 4α-PDD exerted no effect. Since this PDD effect on Cx43 content was sensitive to SB203580 this indicates involvement of p38 MAP-kinase. In contrast MEK-1 (although this pathway in principle can be activated via PKC) does not seem to contribute to PDD-dependent regulation of Cx43 as became evident from the lack of effect of PD98059. An enhancement of Cx43 following PKC activation has also been observed in other cells, although the subsequent signal transduction cascade seems to depend on the cell type. Thus, in human uterine smooth muscle PKC activation (with 12-O-tetradecanoylphorbol 13-acetate (TPA)) led to an increase in Cx43 via c-Fos and c-Jun the molecular constitutes of transcription factor AP-1 (activating protein-1)[36]. Accordingly, an AP-1 site has been identified in the Cx43 promotor[37,38].

Moreover, in neonatal rat cardiomyocytes, stimulation of ET_A or AT_1-receptors (which lead to PKC activation) has been shown to lead to enhanced Cx43 content[13]. These effects were transduced via ERK1/2 (endothelin) or both ERK1/2 and p38-MAP-kinase (angiotensin-II), the latter supporting present finding of PKC/p38 MAP-kinase-dependent Cx43 regulation.

The sensitivity of the observed increases in Cx43 protein to p38 inhibition by SB203580 indicated a possible regulation on the transcriptional level. This is supported by present PCR findings which showed in parallel to the increases in Cx43 protein enhanced Cx43-mRNA signals which also were inhibited by SB203580. Thus, it can be concluded, that the changes seen in this study regarding Cx43 resemble changes on the transcriptional level.

In contrast to Cx43 the connexin 40 seems not to be regulated by stimulation of the PKA, since administration of either forskolin or db-cAMP did not alter the Cx40 content. Moreover, in PCR studies we have not seen any change in Cx40 mRNA relative to the housekeeping gene GAPDH. In contrast, the PKC aktivator PDD led to a marked increase in Cx40 protein. Suprisingly, the PCR studies revealed no change in Cx40 mRNA following administration of PDD thus indicating that the rise in Cx40 is probably not due to *de novo* synthesis or transcriptional regulation by PDD. Thus, the underlying mechanism for this observation remains unclear at present.

Taken together, present results are in support of the hypothesis that in neonatal cardiomyocytes activation of p38 MAP kinase is a common pathway leading to increase in Cx43 content. This pathway seems to be activated by stimulation of AC/cAMP/PKA pathway or PKC signaling pathway. This may be of pathophysiological importance for cardiac diseases and opens possibilities for new pharmacological strategies for control of connexin content.

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