# 4. RESULTS

## 4.1 Establishment of the H9c2 cardiomyoblast cell model

In order to study the expression regulation of the human ALC-1 (hALC-1) gene, a reporter gene construct was generated consisting of the luciferase reporter gene under the control of the hALC-1 promoter and a selection marker. Transfection of the H9c2 cardiomyoblasts with this reporter gene construct resulted in a stable cell line (H9c2T1), which served as a model (Fig. 4) to analyse factors potentially regulating the hALC-1 promoter. Quantification of the luciferase level in the H9c2T1 cardiomyoblasts with a corresponding assay (luciferase assay) then allowed to draw conclusions concerning the activity of the hALC-1 promoter.

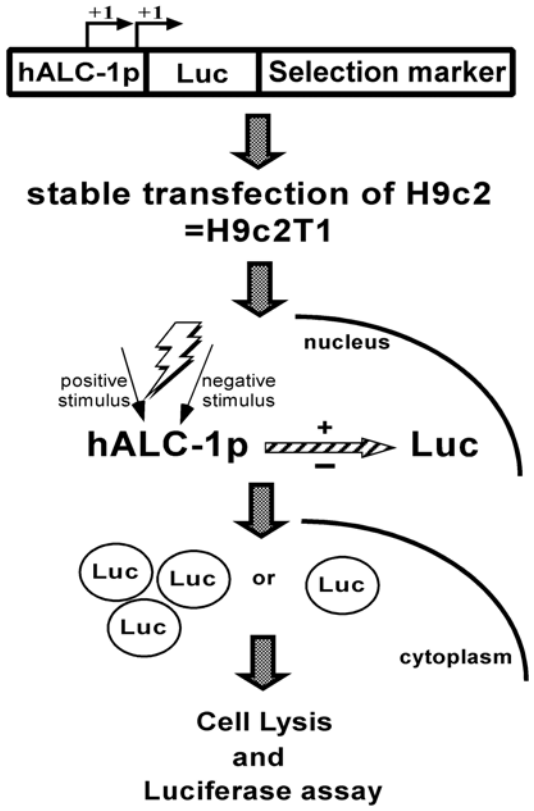


Figure 4. Scheme of the established cell model. Stable transfection of the H9c2 cardiomyoblasts with the reporter gene construct led to the establishment of the H9c2T1 cell line. This cell line then served as a model to characterize the regulation of the human ALC-1 promoter. Activation (positive stimulus) or repression (negative stimulus)

of the promoter led to enhanced (+) or lowered (-) transcription of the luciferase gene and a subsequent increase or decrease in synthesis of the protein, which could be analyzed using a luciferase assay. Therefore, the amount of luciferase was a measure for the state of activity of the hALC-1 promoter. hALC-1p: human ALC-1 promoter; Luc: luciferase reporter gene (in reporter gene construct and in cell nucleus) or reporter protein (in cytoplasm; circled), respectively.

### 4.1.1 Generation of the reporter gene construct

The hALC-1 promoter was amplified from human genomic DNA by PCR (-1942 bp to +75 bp) and verified by DNA sequencing. In the next step, a construct was generated consisting of the amplified hALC-1 promoter cloned in front of the luciferase gene as a reporter gene. For selection of the transfected cells, a resistance cassette including the SV40 promoter and the selection marker neomoycine (Neo), was also added, next to other eukaryotic processing elements, leading to the reporter gene construct hALC-1 promoter-Luciferase-Neo (Fig. 5A).

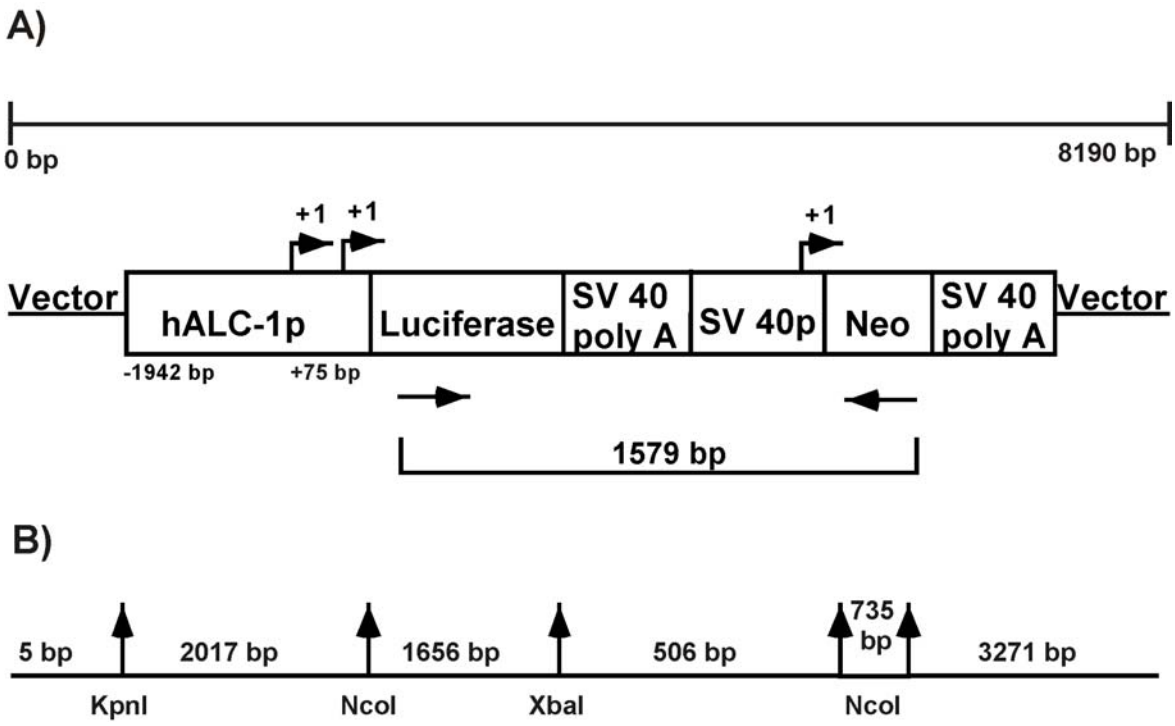


Figure 5. Scheme of the reporter gene construct. A): Vector: luciferase reporter gene vector; hALC-1p: human ALC-1 promoter (-1942 to + 75 bp); Luciferase: luciferase

reporter gene; SV40p: SV40 promoter; SV40 polyA: SV40 polyadenylation signal; Neo: neomycine resistance gene; +1: transcription start site; lower arrows indicate location of primers used for genotyping of the stably transfected H9c2T1 cardiomyoblast line by PCR, leading to an amplification product of 1579 bp. Size of the complete reporter gene construct: 8190 bp. B): Overview of the restriction enzyme sites of KpnI, NcoI and XbaI within the reporter gene construct and the corresponding restriction fragments (in bp).

The hALC-1 promoter within the reporter gene construct was verified by sequence analysis and subsequent alignment with the published sequence (cf. Appendix 1). Also, the reporter gene construct was analyzed with several restriction enzymes (cf. Fig. 5B). Figure 6 shows the gel electrophoresis of the digested DNA revealing the correct band pattern.

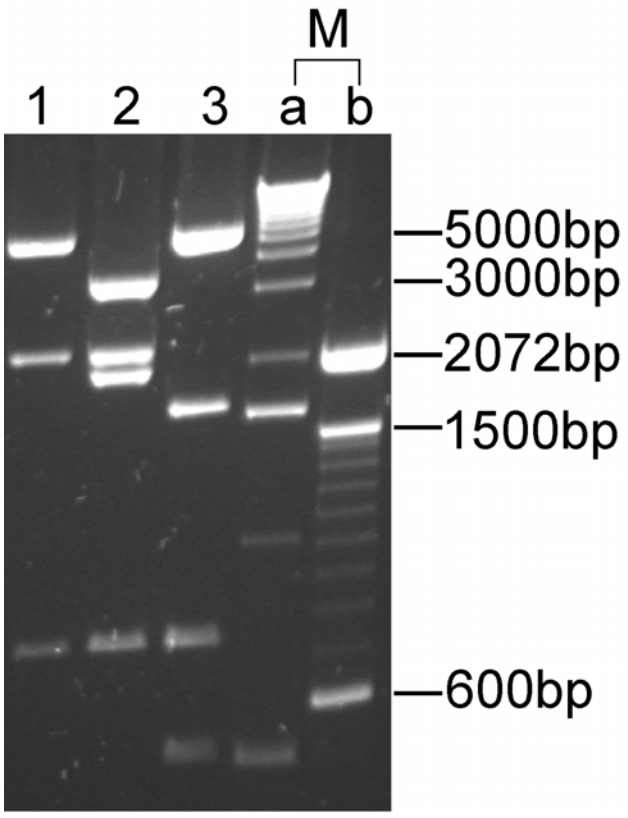


Figure 6. Restriction enzyme analysis of the reporter gene construct. Digestion of the reporter gene construct with NcoI (lane 1), NcoI and KpnI (lane 2) and NcoI and XbaI (lane 3) and subsequent gel electrophoresis gave the predicted band pattern: lane 1: 735 bp, 2162 bp and 5293 bp; lane 2: 735 bp, 2017 bp, 2162 bp and 3276 bp; lane 3:

506 bp, 735 bp, 1656 bp and 5293 bp (cf. Fig. 5B). M: marker; Ma: 1 Kb DNA ladder; Mb: 100 bp DNA ladder (band sizes cf. 2.7).

### 4.1.2 Generation of the stably transfected H9c2 cardiomyoblast lines (H9c2T1 and H9c2T2)

Before attempting to generate a stable cell line, different transfection methods and parameters were tested. Integration of DNA into the chromosome occurs with low frequency. Moreover, transfection efficiency varies greatly among different cell types and transfection methods (Ausubel et al. 2003). Therefore, a variety of transfection techniques were analyzed for their efficiency when applied in H9c2 cardiomyoblasts (Table 2). After optimization of parameters such as the amount and quality of DNA used, the cell confluency at the time of transfection and the duration of transfection, the calcium phosphate precipitation method was found to be most effective and used in order to transfect the hALC-1 promoter-Luciferase-Neo reporter gene construct into the H9c2 cardiomyoblasts.

| Transfection kit | Transfection method | Efficiency of transfection |
|---|---|---|
| Calcium Phosphate Transfection System (Invitrogen Life Technologies) | Calcium phosphate precipitation | around 25% |
| GeneShuttle™ Transfection Reagent (Q Biogene) | Liposome based transfection | around 2% |
| GenePorter™ Transfection Reagent (Gene Therapy Systems Inc.) | Liposome based transfection | around 5% |
| Nucleofector Kit (Amaxa) | Electroporation | around 10% |

Table 2. Overview of the different tested transfection methods and of their efficiency in H9c2 cardiomyoblasts.

The transfectants were selected with G418 (geneticin), leading to their survival and growth after a few days (Fig. 7, lower panel). However, wild type cells could not grow in the presence of G418 and died (Fig. 7, upper panel). Morphological differences between the wild type H9c2 cardiomyoblasts and the stably transfected H9c2T cells were not apparent. Subcloning of the surviving cells led to the cell lines H9c2T1 and H9c2T2.

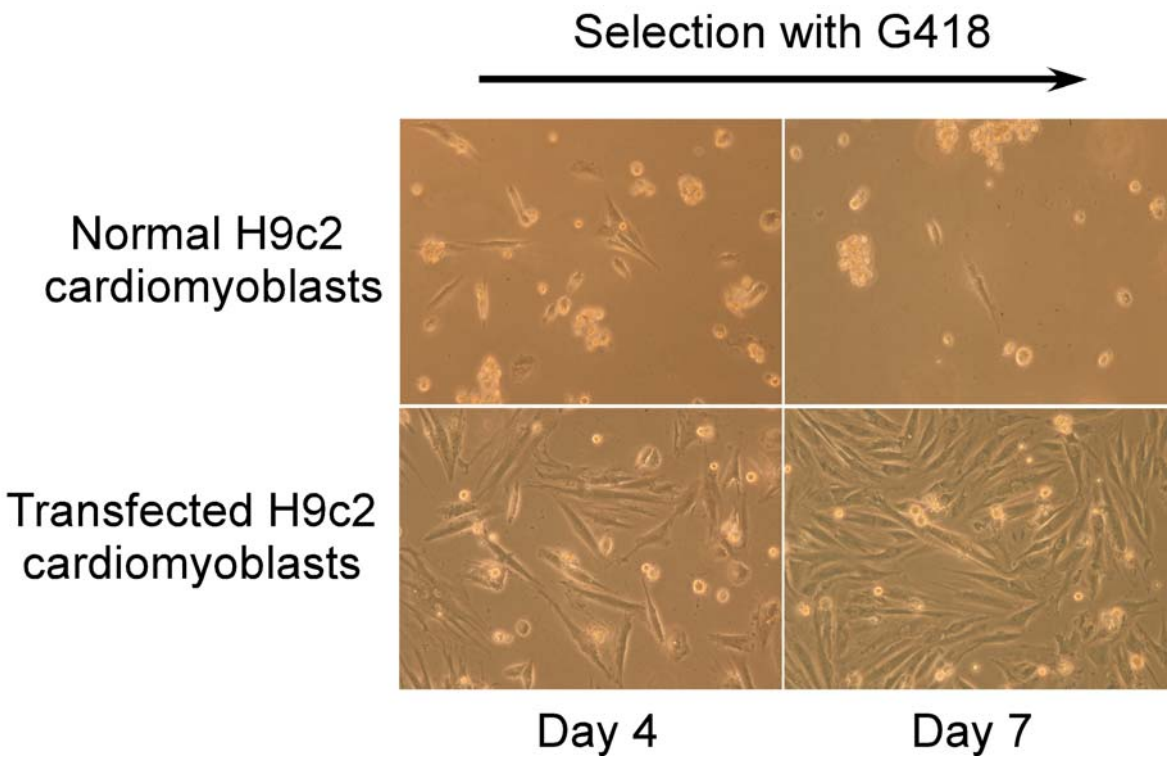


Figure 7. Generation of the stably transfected H9c2 cardiomyoblasts. H9c2 wild type cardiomyoblasts were transfected with the reporter gene construct using the calcium phosphate precipitation method. Selection with G418 led to cell death of the untransfected H9c2 cardiomyoblasts (upper panel), whereas the transfected cells survived (day 4-lower panel) and began to multiply after a few days (day 7-lower panel) (magnification 10-fold).

To verify the integration of the reporter gene construct into the genomic DNA of the stably transfected cells, DNA was isolated from H9c2T1 and wild type H9c2 cardiomyoblasts and a PCR was performed using construct specific primers (cf. arrows Fig. 5A). Subsequent gel electrophoresis (Fig. 8) showed the specific amplification product (1579 bp) using the construct itself as a positive control (lane 1), and DNA

obtained from H9c2T1 cardiomyoblasts (lane 3). As expected, DNA prepared from wild type H9c2 cardiomyoblasts showed no amplification product (lane 2).

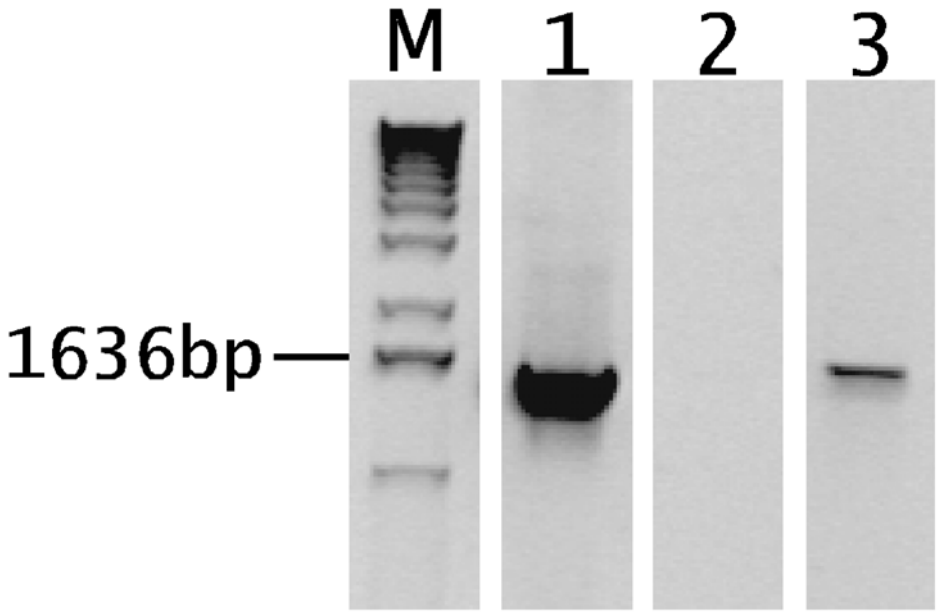


Figure 8. Genotyping of the H9c2T1 cell line. Genomic DNA was extracted from H9c2 and H9c2T1 cardiomyoblasts. Using specific primers, a PCR was performed, and subsequent gel electrophoresis showed a 1579 bp fragment amplified in the positive control (reporter gene construct, lane 1), in the H9c2T1 cardiomyoblasts (lane 3) but not in the wild type H9c2 cardiomyoblasts (lane 2); M: marker.

In order to further characterize the stably transfected H9c2T1 cardiomyoblasts, their proliferation rate was compared with that of the wild type H9c2 cardiomyoblasts. To that end, the number of living cells was determined every 24 hours for a period of 4 days, which is the time frame of a vasopressin experiment. Figure 9 shows that the growth rates of the two cell lines did not significantly differ from each other.

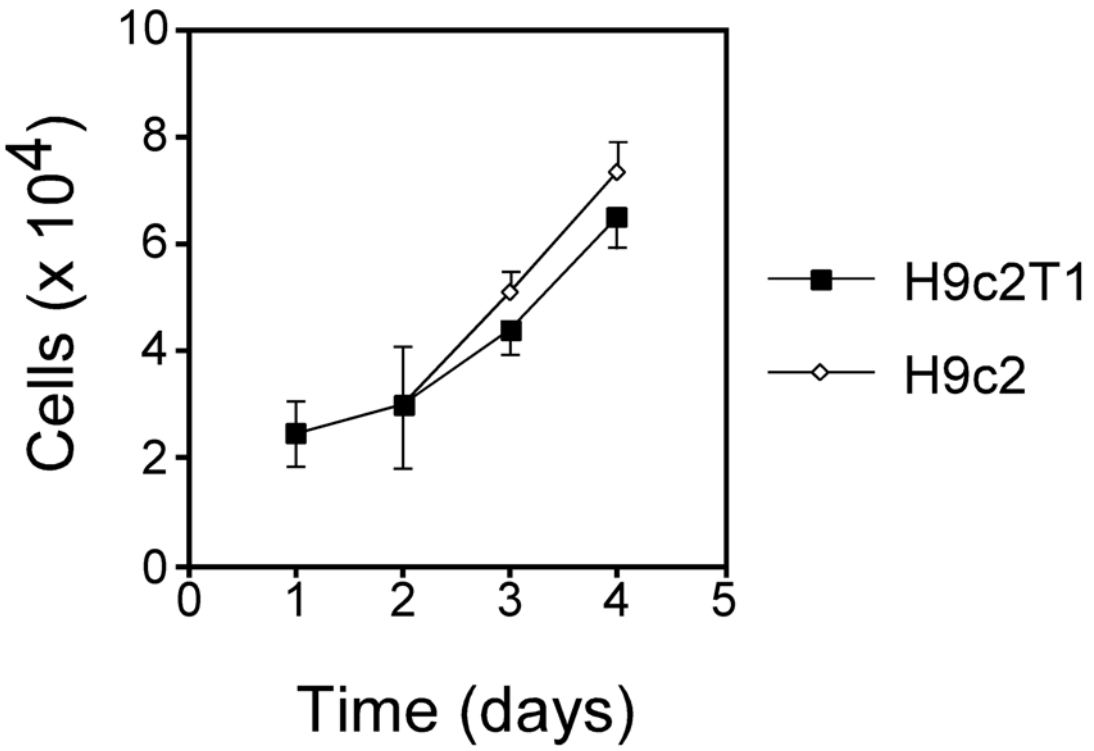


Figure 9. Proliferation curve. 2,5 x $10^4$ H9c2 or H9c2T1 cardiomyoblasts were seeded in duplicates. The number of living cells (n=2) was determined every 24 hours for 4 days. Bars are means ± SEM.

### 4.1.3 Establishment of the luciferase assay

The luciferase in the cellular extracts was measured with a luciferase assay. It is based on the enzymatic activity of luciferase, which catalyzes the oxidation of beetle luciferin and thereby leads to the production of light (Ausubel et al. 2003). This luminescence was analyzed after programming a luminometer with specific parameters. Using the luminescence of a recombinant protein, a calibration curve was generated which allowed the quantification of the extracted luciferase (Fig. 10).

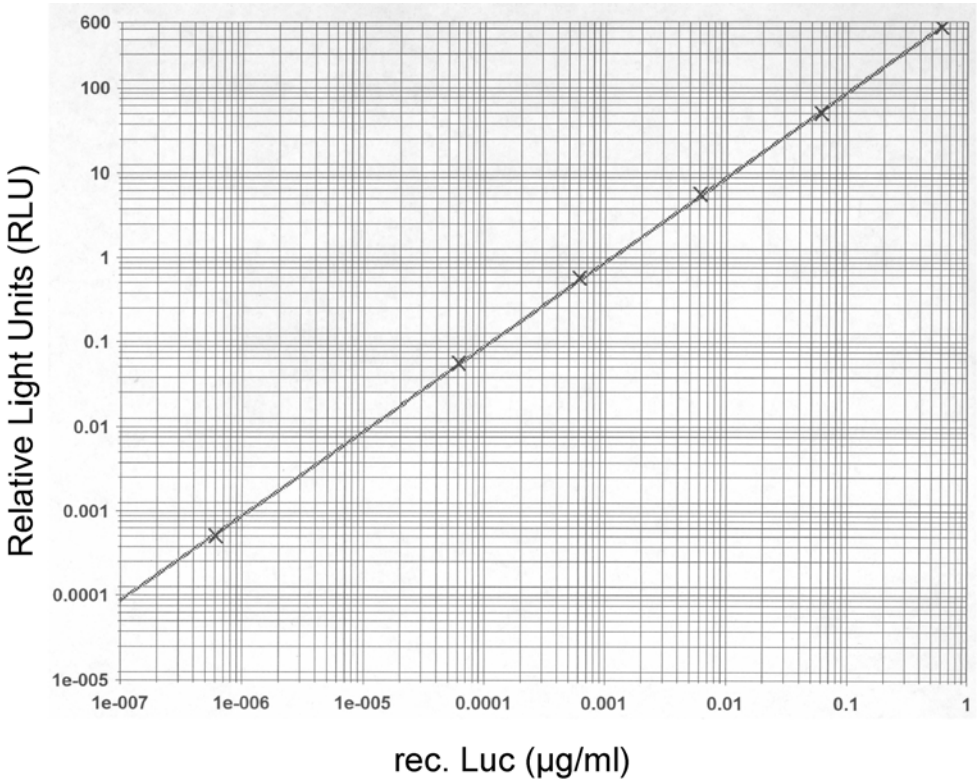


Figure 10. Calibration curve for the luciferase assay. Measuring the luminescence of a recombinant luciferase (rec. Luc) in relative light units (RLU), a calibration curve for the calculation of the amount of luciferase (µg/ml) was obtained.

### 4.2 Stimulation of H9c2T1 cardiomyoblasts with vasopressin

H9c2 cardiomyoblasts express a $V_{1a}$ receptor. Stimulation with vasopressin is known to activate a panel of signaling pathways and to lead to hypertrophy in these cells, which is demonstrated by an overall increase in protein synthesis (Chen and Chen 1999; Brostrom et al. 2000).

Treatment with vasopressin was performed under conditions of serum starvation. In order to avoid differentiation of the H9c2T1 cardiomyoblasts into myotubes under these conditions (Kimes and Brandt 1976), cell densitiy was always kept beneath confluency. Visual inspection of the cardiomyoblasts during the experiment confirmed the absence of myotubes.

Unstimulated H9c2T1 cardiomyoblasts revealed a well-detectable luciferase signal even under serum-free conditions (Fig. 11), suggesting the presence of a basal activity of the hALC-1 promoter. The amount of luciferase increased 4.4-fold upon addition of vasopressin (1 μM) (Fig. 11), indicating that vasopressin stimulation of the H9c2T1 cardiomyoblasts leads to a statistically significant ($p<0.001$) activation of the hALC-1 promoter.

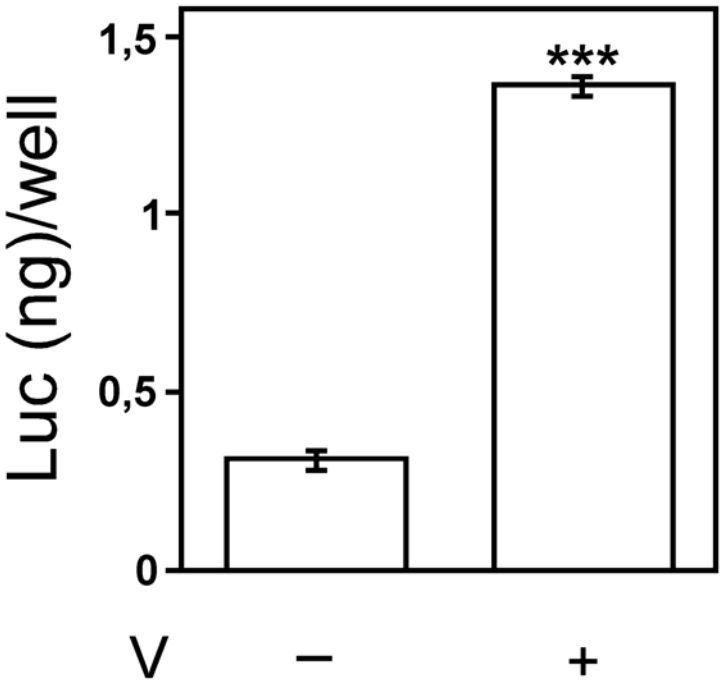


Figure 11. Vasopressin treatment of the H9c2T1 cardiomyoblasts. H9c2T1 cardiomyoblasts were seeded and 24 hours later serum starvation was induced, which was maintained for the remainder of the experiment. After another 24 hours, H9c2T1 cardiomyoblasts were treated with 1μM vasopressin (V) for 48 hours and the amount of luciferase (Luc) expressed as ng luciferase/well was analyzed. Treated H9c2T1 cardiomyoblasts (V+) revealed a 4.4-fold higher amount of luciferase in comparison with untreated cells (V-), suggesting an enhanced hALC-1 promoter activity (in vasopressin-stimulated cells). Bars are means ± SEM of triplicate experiments. Significance analysis was performed using the Student's t-test. *** $p<0.001$ (compared versus V-).

In order to confirm a hypertrophic response of H9c2T1 cardiomyoblasts upon stimulation with vasopressin, the amount of protein of 6 cell cultures was quantified.

Compared with untreated H9c2T1 cardiomyoblasts, the protein content rose significantly ($p<0.05$) in vasopressin-stimulated H9c2T1 cardiomyoblasts (Fig. 12).

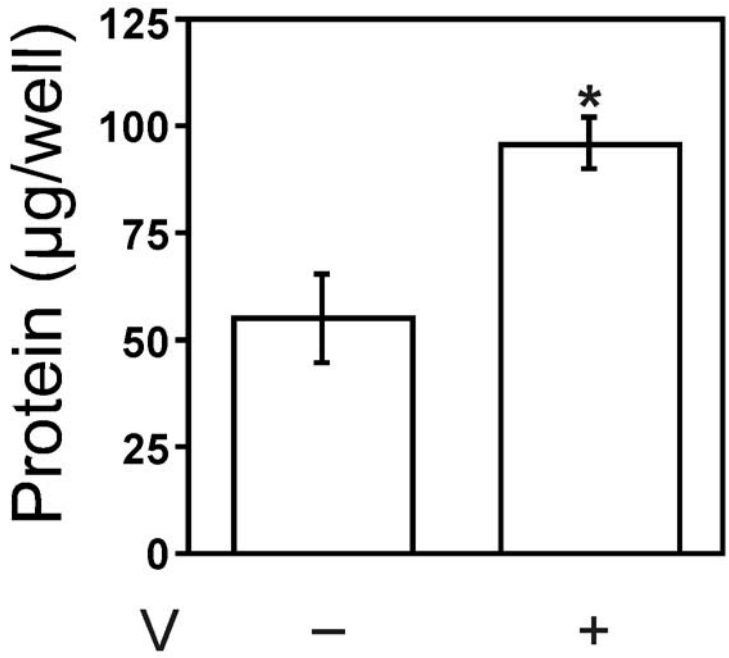


Figure 12. Vasopressin induces hypertrophy in H9c2T1 cardiomyoblasts. H9c2T1 cardiomyoblasts were stimulated with vasopressin (1 μM). After 48 hours the cells were harvested and the amount of protein (μg/well) was analyzed. Treated H9c2T1 cardiomyoblasts (V+) revealed a 1.7-fold protein content in comparison with untreated cells (V-), suggesting that hypertrophy of the H9c2T1 cardiomyoblasts had taken place. Around 2.5 x $10^5$ cells were counted per well in unstimulated as well as stimulated cell cultures. Bars are means ± SEM of triplicate experiments. Significance analysis was performed using the Student's t-test. * $p<0.05$ (compared versus V-).

### 4.3 Analysis of endogenous ALC-1 expression in H9c2T1 cardiomyoblasts

In order to analyze endogenous rat ALC-1 expression in unstimulated and vasopressin-stimulated H9c2T1 cardiomyoblasts, protein was extracted and subjected to Western blot analysis. Two different antibodies were used for immunoblotting: 1) an isoform-specific antibody raised against rat ALC-1 (Fig. 13.1) and 2) a panspecific ALC-1 antibody (Fig. 13.2.) raised against a region in human ALC-1, which is highly

homologous in rat and human ALC-1 as well as VLC-1 (cf. 3.10.1). The isoform-specific antibody reacted selectively with the rat ALC-1 in a SDS-protein extract of rat atrium (positive control) but not of rat ventricle (negative control). Using the same antibody, the rat ALC-1 could not be detected in unstimulated rat H9c2T1 cardiomyoblasts. In addition, expression of the endogenous rat ALC-1 gene could not be induced in the cells upon vasopressin (1 μM) stimulation (Fig. 13.1). Using the panspecific ALC-1 antibody, both rat ALC-1 and rat VLC-1 could be detected due to their homologous regions. Using this panspecific antibody, endogenous rat ALC-1 or VLC-1 could not be detected in the H9c2T1 cardiomyoblasts. In accordance with the results obtained with the isoform-specific antibody, vasopressin stimulation did not induce endogenous rat ALC-1 or VLC-1 expression (Fig. 13.2).

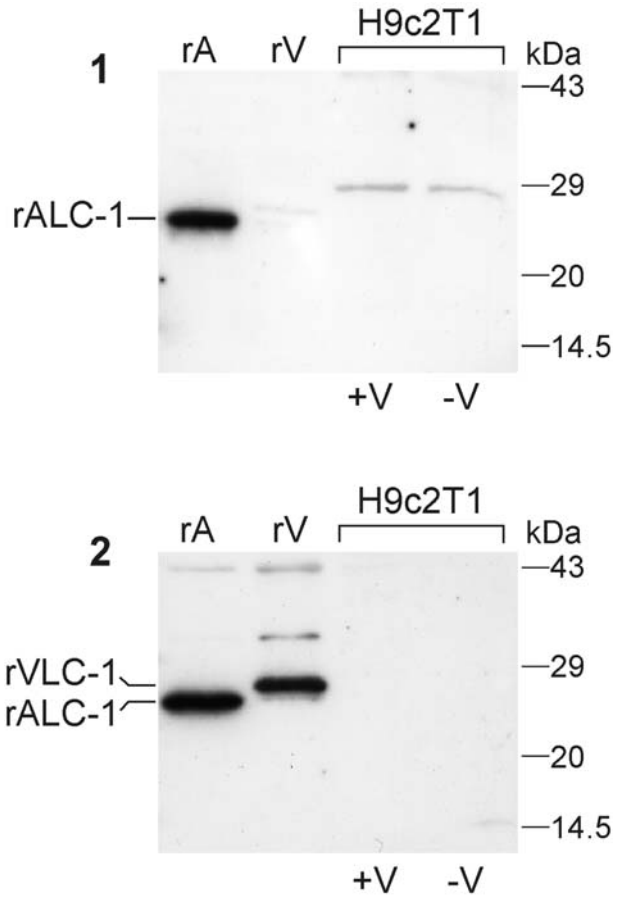


Figure 13. Western blot analysis of endogenous ALC-1 in H9c2T1 cardiomyoblasts. Protein was extracted from unstimulated (-V) and vasopressin-stimulated (1 μM) (V+) H9c2T1 cardiomyoblasts as well as from rat atrium (rA; positive control) and rat ventricle (rV; negative control). The samples were subjected to SDS-PAGE and

subsequent immunoblotting using an isoform-specific antibody raised against rat ALC-1 (13.1.) or a panspecific ALC-1 antibody (13.2.). Endogenous rat ALC-1 (rALC-1) or rat VLC-1 (rVLC-1) could not be detected in vasopressin-treated or untreated H9c2T1 cardiomyoblasts using either antibody.

## 4.4 Analysis of the involved $Ca^{2+}$ sources

### 4.4.1 Treatment of H9c2T1 cardiomyoblasts with ionomycin

H9c2T1 cardiomyoblasts were treated with ionomycin. This calcium ionophore leads to a rise in intracellular $Ca^{2+}$ by transporting $Ca^{2+}$ from the extracellular into the intracellular space, as well as by mobilizing intracellular sequestered $Ca^{2+}$ (Liu and Hermann 1978; Albert and Tashjian 1984 and 1986). The aim of this experiment was to mimic vasopressin-induced alterations in $Ca^{2+}$. H9c2T1 cardiomyoblasts were treated with 0.5 μM ionomycin for 30 min. The cardiomyoblasts were then depleted of ionomycin by washing with pure medium containing 5% BSA. Normal culture medium was added and the cells were harvested for luciferase analysis 48 hours later. Figure 14 shows that the luciferase level is significantly higher in ionomycin-treated cells (left column) compared with untreated cells (right column). This suggests that the hALC-1 promoter is activated by an increase in free intracellular $Ca^{2+}$.

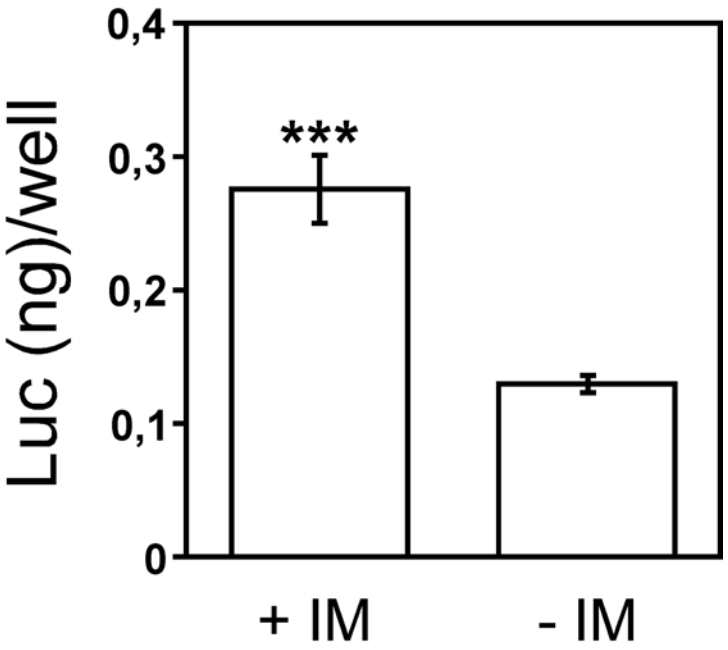


Figure 14. Treatment of H9c2T1 cardiomyoblasts with ionomycin. H9c2T1 cardiomyoblasts were treated with 0.5 μM ionomycin (IM) for 30 min. After 48 hours, the cells were harvested and a luciferase assay was performed. Ionomycin-treated cells (+ IM) showed a significantly higher (***, $p < 0.001$) luciferase level than untreated cells (- IM), indicating a stimulation of the hALC-1 promoter activity under conditions of increased intracellular $Ca^{2+}$. Bars are the means ± SEM of triplicate experiments. Significance analysis was performed using the Student's t-test.

### 4.4.2 Treatment of H9c2T1 cardiomyoblasts with vasopressin in $Ca^{2+}$-free culture medium

H9c2T1 cardiomyoblasts were stimulated with vasopressin in normal culture medium or in medium nominally lacking $Ca^{2+}$. A subsequent assay did not reveal any significant changes in the amount of luciferase (Fig. 15). This indicates that intracellular rather than extracellular $Ca^{2+}$ sources are involved in the stimulation of the hALC-1 promoter under vasopressin-induced hypertrophic conditions.

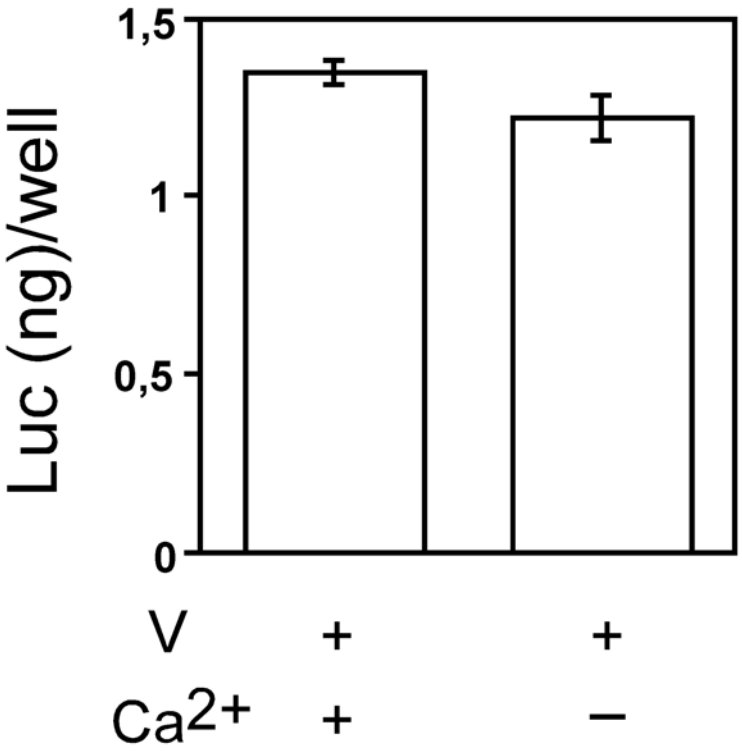


Figure 15. Role of $Ca^{2+}$ sources in vasopressin-treated H9c2T1 cardiomyoblasts. H9c2T1 cardiomyoblasts were stimulated with vasopressin (1 μM) and cultured in medium with or without $Ca^{2+}$. A luciferase assay revealed no significant changes in the amount of luciferase, suggesting that extracellular $Ca^{2+}$ sources are not involved in the vasopressin-mediated activation of the hALC-1 promoter. V+, with vasopressin; V-, without vasopressin; + $Ca^{2+}$, cultured in the presence of $Ca^{2+}$; - $Ca^{2+}$, cultured without $Ca^{2+}$. Bars are the means ± SEM of triplicate experiments.

### 4.5 Analysis of hypertrophic signaling pathways

Vasopressin stimulation of H9c2 cardiomyoblasts leads to the activation of a variety of signaling cascades (Chen and Chen 1999). In further experiments, those signaling pathways which potentially play a role in cardiac hypertrophy were studied using pharmacological inhibitors and immunofluorescence analysis. These include protein kinase C, the $Ca^{2+}$-calmodulin-NFAT pathway and the $Ca^{2+}$-calmodulin-dependent protein kinases.

### 4.5.1 Inhibition with bisindolylmaleimide

To elucidate the involvement of PKC in the regulation of the hALC-1 promoter, the cell-permeable protein kinase inhibitor bisindolylmaleimide (BIM) was used in two different concentrations (200 and 400 nM). BIM binds competitively at the ATP binding site in the catalytic domain of the PKC. It is highly selective for the inhibition of PKC and inhibits membrane and cytosolic PKC with similar potencies. BIM is especially useful, since the 200 nM dose completely inhibits the $\alpha$ and $\beta$ PKC isoform, whereas the 400 nM dose leads to a considerable repression of the $\delta$ and $\varepsilon$ isoforms (Martiny-Baron et al. 1993; Budworth and Gescher 1995; Toullec et al. 1991).

H9c2T1 cardiomyoblasts were pretreated with BIM in the two different concentrations for 1 hour before vasopressin was added (1 $\mu$M) and remained in the medium during the experiment. After 48 hours, the cardiomyoblasts were harvested and the amount of luciferase was determined with the luciferase assay. Vasopressin treatment of the cardiomyoblasts led to a significant rise in luciferase (Fig. 16: –V versus + V; $p<0,001$). However, no change could be detected in luciferase activity when vasopressin-stimulated H9c2T1 cardiomyoblasts were additionally pretreated with 200 nM BIM (Fig. 16: + V versus +V/+ 200 nM BIM). The higher concentration of 400 nM BIM led to a minor but statistically non-significant decrease in luciferase activity compared with H9c2T1 cardiomyoblasts solely stimulated with vasopressin (Fig. 16: +V versus +V/+ 400 nM BIM). This suggests that the different PKC isoforms are not involved in the stimulation of the hALC-1 promoter under hypertrophic conditions.

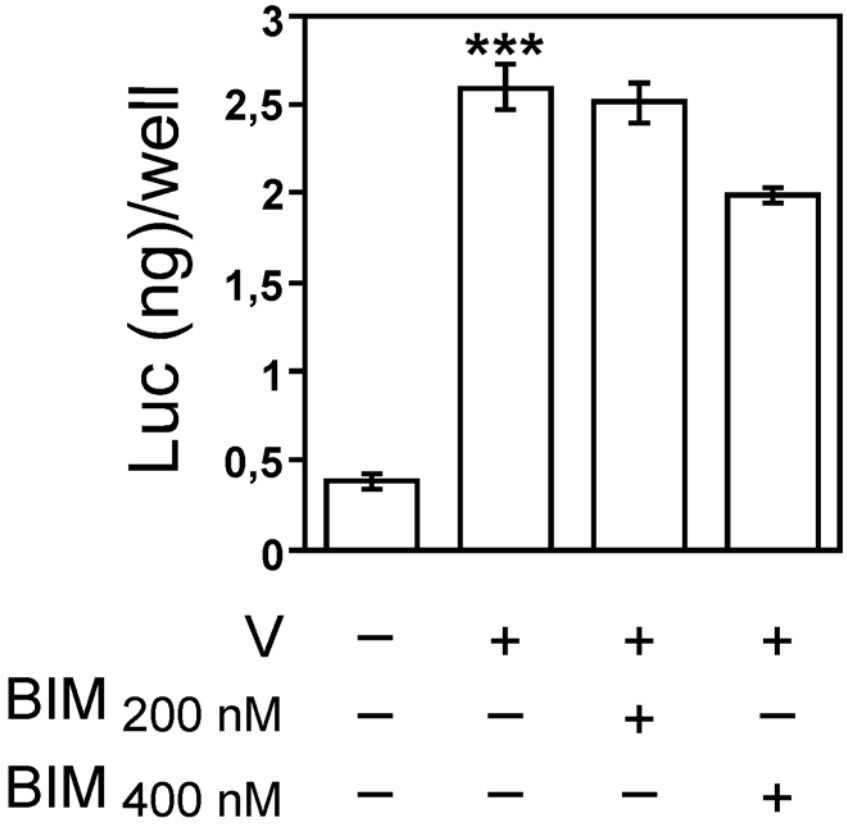


Figure 16. Treatment of the H9c2T1 cardiomyoblasts with the PKC inhibitor bisindolylmaleimide. Treatment of H9c2T1 cells with vasopressin (1 μM) led to a significant rise in the luciferase level. Additional pretreatment with 200 nM or 400 nmM BIM did not siginificantly change the luciferase signals, indicating that the PKC does not play a role in activation of the hALC-1 promoter under vasopressin stimulation. V, vasopressin; BIM, bisindolylmaleimide; +, treated; -, untreated. Bars are the means ± SEM of triplicate experiments. Significance analysis was performed using ANOVA and Bonferroni as a post test. *** $p<0.001$ (compared with V-, BIM-).

### 4.5.2 Analysis of the $Ca^{2+}$-calmodulin-calcineurin-NFAT pathway

The $Ca^{2+}$-calmodulin-calcineurin-NFAT pathway plays an important role in the regulation of hypertrophic genes (Molkentin et al. 1998). Therefore, the involvement of this pathway in the activation of the hALC-1 promoter was analyzed.

#### 4.5.2.1 NFAT localization in H9c2T1 cardiomyoblasts

The binding of $Ca^{2+}$ to calmodulin leads to its activation. This $Ca^{2+}$-calmodulin complex in turn activates the phosphatase calcineurin, which dephosphorylates the transcription factor NFAT, leading to its translocation to the nucleus where it can act as a transcription factor. Among the different NFAT family members, NFAT3 is highly expressed in cardiac tissue and is also involved in cardiac hypertrophy (cf. 1.1.3.2.1). In order to determine whether NFAT3 translocation takes place in response to vasopressin, H9c2T1 cardiomyoblasts were stimulated with vasopressin (1 μM) and NFAT localization was analyzed after immunofluorescence staining of the cells with an antibody raised against NFAT3 (Fig. 17). Also, the percentage of nuclear NFAT3 staining was determined (Fig. 18).

In untreated H9c2T1 cardiomyoblasts, NFAT3 was predominantly localized in the cytoplasm (Fig. 17A), while only 7 % of the nuclei contained NFAT3 (Fig. 18 left column). However, upon vasopressin stimulation, NFAT staining was found almost exclusively in the nucleus of the cells (Fig. 17B and Fig. 18 right column). This demonstrates that NFAT3 translocation from the cytoplasm to the nucleus takes place in response to vasopressin stimulation.

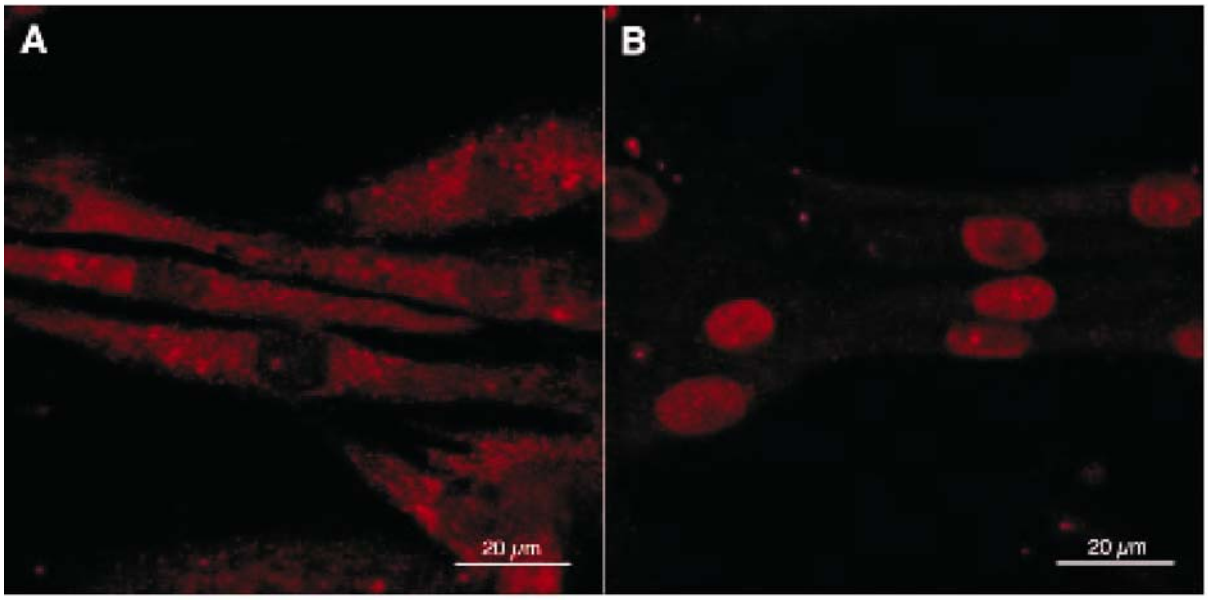


Figure 17. Analysis of NFAT3 localization in H9c2T1 cardiomyoblasts. Immunofluorescence of H9c2T1 cardiomyoblasts was carried out using the anti-NFAT3 antibody as primary antibody visualized by Cy3. Untreated cells (A) showed a mainly cytoplasmic localization of NFAT3, whereas vasopressin stimulation (1 µM) of the cells (B) led to an almost exclusive nuclear staining of the cells.

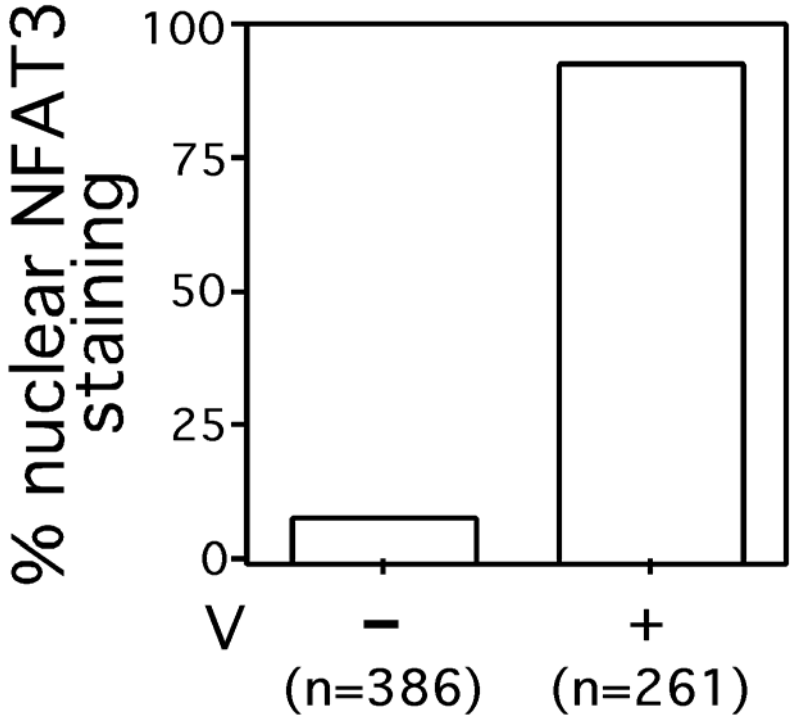


Figure 18. Percentage of nuclear NFAT3 staining in H9c2T1 cardiomyoblasts. While only 7% of the nuclei contained NFAT3 in untreated cells (left column, V-), almost all

nuclei (93%) became NFAT3 positive upon vasopressin stimulation (1 μM) of H9c2T1 cardiomyoblasts (right column, V+).

#### 4.5.2.2 Specificity of the NFAT antibody

In order to confirm the specificity of the NFAT3 antibody, experiments using a specific blocking peptide were performed.

A typical vasopressin experiment was performed. The untreated or vasopressin-treated (1 μM) H9c2T1 cardiomyoblasts were incubated with the primary antibody against NFAT3 as well as with a blocking peptide, and immunufluorescence was carried out as before (cf. 4.5.2.1). In addition, in order to show the nuclei of the cells, DAPI staining was performed. Figure 19a shows the predominant cytoplasmic staining of untreated H9c2T1 cardiomyoblasts (A) and their almost omitted nuclei. DAPI staining of these cells emphasizes this effect (B). When the untreated cells were additionally incubated with the blocking peptide, the NFAT3 staining is nearly absent (C) and only the nuclei are visualized by DAPI (D). In vasopressin-stimulated H9c2T1 cardiomyoblasts (Fig. 19b), a mainly nuclear staining (A and B) is almost completely obliterated in the presence of blocking peptide (C and D). These data confirm the specificity of the anti-NFAT3 antibody.

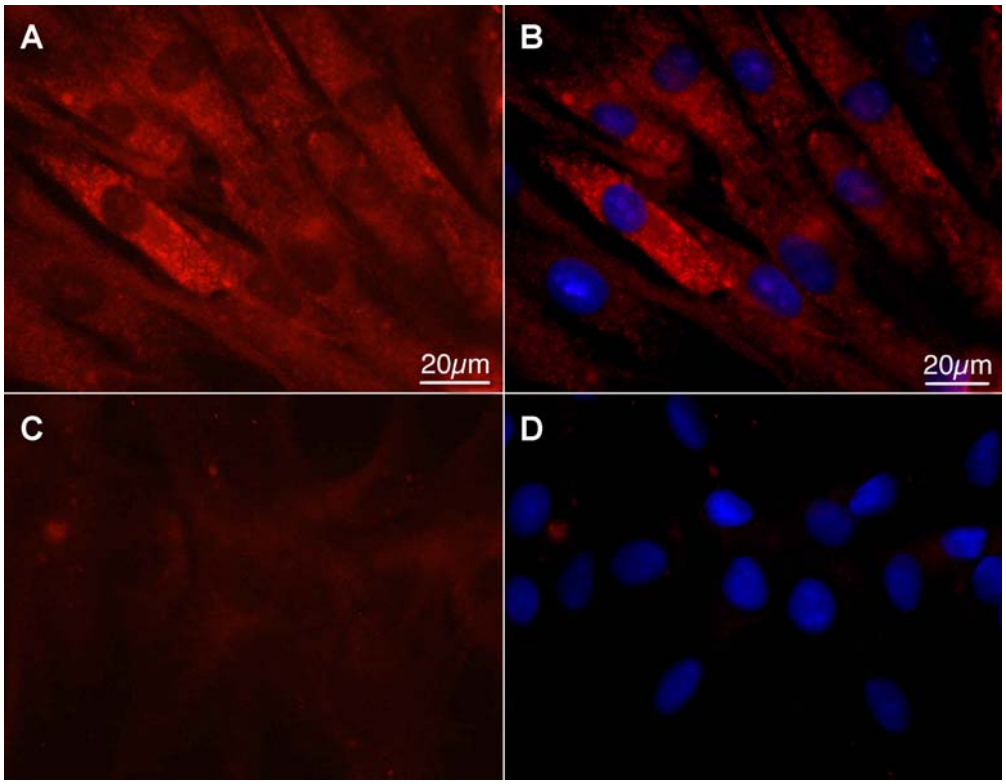


Figure 19a. Specificity of the NFAT3 antibody in untreated H9c2T1 cardiomyoblasts. Unstimulated H9c2T1 cardiomyoblasts were harvested for immunofluorescence analysis and incubated with the anti-NFAT3 antibody (A-D) and additionally with a specific blocking peptide (C and D). Visualization of the nuclei was performed by DAPI staining (B and D). The predominant cytoplasmic NFAT3 staining is nearly absent when the blocking peptide is used, confirming the specificity of the antibody.

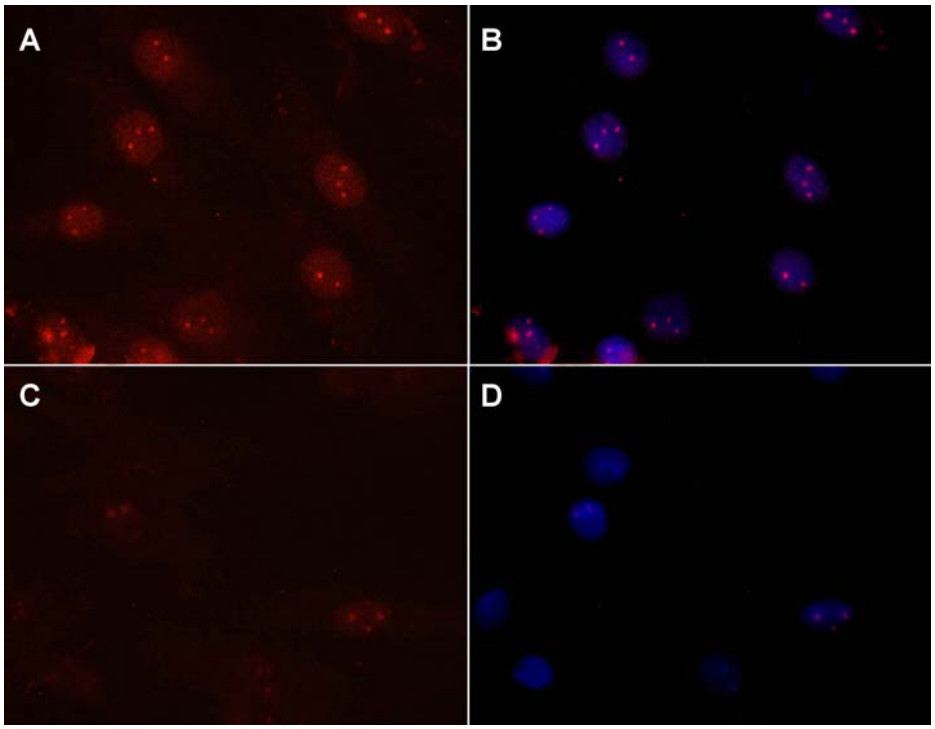


Figure 19b. Specificity of the NFAT3 antibody in vasopressin-treated H9c2T1 cardiomyoblasts. Vasopressin (1 μM)-stimulated H9c2T1 cardiomyoblasts were harvested for immunofluorescence analysis. Incubation with the anti-NFAT3 antibody (A-D) and with a specific blocking peptide (C and D) followed. DAPI staining was used to show the nuclei (B and D). The mainly nuclear NFAT3 staining almost disappeared upon additional incubation with the blocking peptide, verifying that the antibody used is specific for NFAT3.

#### 4.5.2.3 Confocal microscopy of NFAT stained H9c2T1 cells

Confocal microscopes selectively collect light from thin optical sections representing focal planes within the specimen. Since light from out-of-focus areas is blocked, structures within the focal plane appear more sharply defined (Ausubel et al. 2003).

The translocation of NFAT from the cytoplasm to the nucleus of vasopressin-stimulated H9c2T1 cardiomyoblasts is a very pronounced effect. In order to exclude that fluorescence from the cytoplasm or the nuclear membrane is a source of false positive stained nuclei, confocal microscopy was applied. A series of different optical sections of NFAT3 stained H9c2T1 cells was analyzed. Figure 19c shows that the staining is not

present in all sections. This confirms that it is the nuclei, and not other structures such as the nuclear membrane, which are the source for the observed fluorescence emission pattern in vasopressin-treated H9c2T1 cardiomyoblasts.

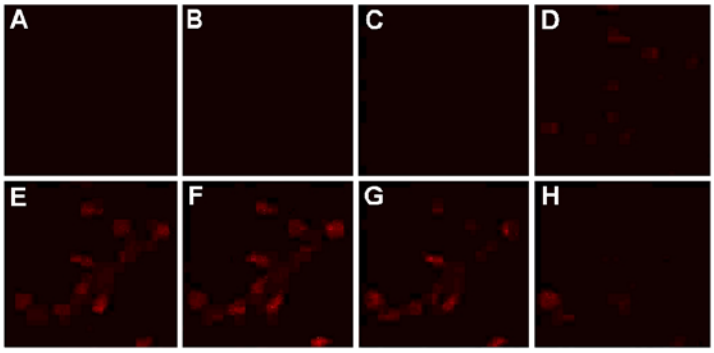


Figure 19c. Confocal microscopy of vasopressin-treated (1μM) H9c2T1 cells, which were stained with fluorescence labelled NFAT3. The different optical sections in series (A-H) verify that the emitted fluorescence signal originates only from the nuclei.

#### 4.5.2.4 Inhibition with cyclosporin A

Nuclear translocation of NFAT requires its dephosphorylation, which is carried out by the activated phosphatase calcineurin (cf. 1.1.3.2.1). Use of an inhibitor can determine the role of calcineurin in the transcriptional upregulation of the hALC-1 promoter after vasopressin stimulation. Cyclosporin A (CspA) inhibits calcineurin by forming a complex with cyclophilin and subsequent binding to the catalytic subunit of calcineurin (Leinwand 2001; Shaw et al. 1995).

Inhibition of vasopressin-stimulated H9c2T1 cardiomyoblasts with the calcineurin inhibitor CspA (1 μM) caused a statistically significant suppression of the luciferase signal compared with untreated vasopressin-stimulated H9c2T1 cardiomyoblasts (Fig. 20). This indicates that calcineurin is involved in vasopressin-induced activation of the hALC-1 promoter and confirms the NFAT translocation data on another level.

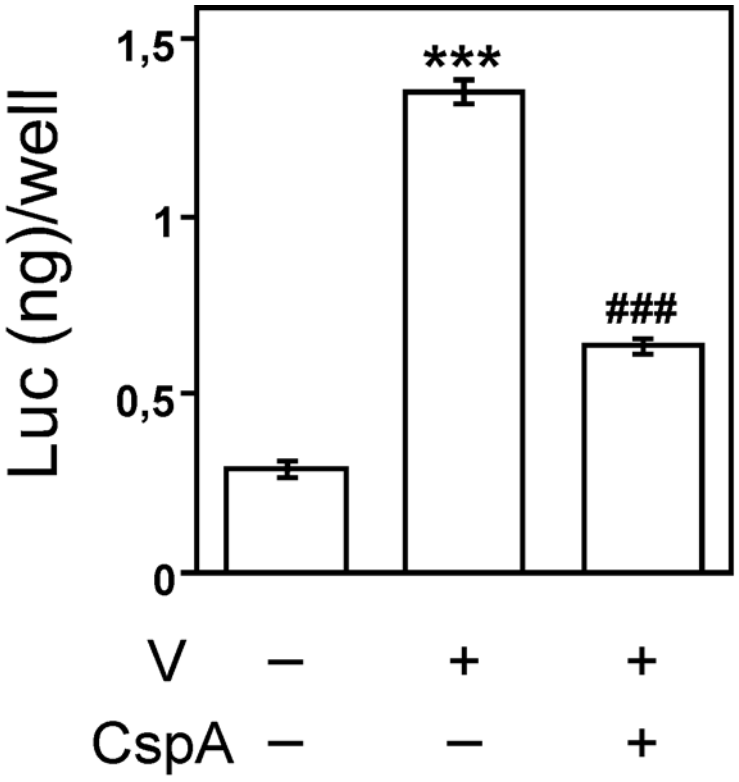


Figure 20. Treatment of the H9c2T1 cardiomyoblasts with the calcineurin inhibitor cyclosporin A. Vasopressin (1μM) stimulation of H9c2T1 cardiomyoblasts caused a 4.6-fold rise in the luciferase level. This was decreased by 53% when the cells were additionally pretreated with cyclosporin A (1 μM). V, vasopressin; CspA, cyclosporin A; +, treated; -, untreated. Bars are means ± SEM of triplicate experiments. Significance analysis was performed using ANOVA and Bonferroni as a post test. *** $p<0.001$ (compared with V-, CspA-); ### $p<0.001$ (compared with V+, CspA-).

### 4.5.3 Analysis of the $Ca^{2+}$-calmodulin-dependent protein kinases (CaMKs)

#### 4.5.3.1 Inhibition with KN93

To study the influence of the multifunctional CaMKs on hALC-1 promoter activation, the membrane permeant inhibitor KN93 was used. KN93 blocks the activity of all CaMKs by competitive inhibition of calmoduline binding to the CaMK (Hook and Means 2001). Its inactive analogue KN92 is a congener of KN93, but without the CaMK inhibitory activity

and is therefore used as an experimental control (Tombes et al. 1995). H9c2T1 cardiomyoblasts were stimulated with vasopressin (1 μM) and pretreated with KN93 or KN92 (10 μM each). Compared with unstimulated H9c2T1 cardiomyoblasts, vasopressin stimulation significantly increased hALC-1 promoter activity. Treatment with KN93 drastically suppressed the vasopressin effect by approximately 80% (Fig. 21) ($p<0.01$). KN92, the inactive KN93 analogue, had no significant influence on the vasopressin effect in H9c2T1 cardiomyoblasts (Fig. 21), confirming the specificity of the KN93-mediated inhibition. These results suggest an important role of the $Ca^{2+}$-calmodulin-dependent protein kinases in the regulation of the hALC-1 promoter.

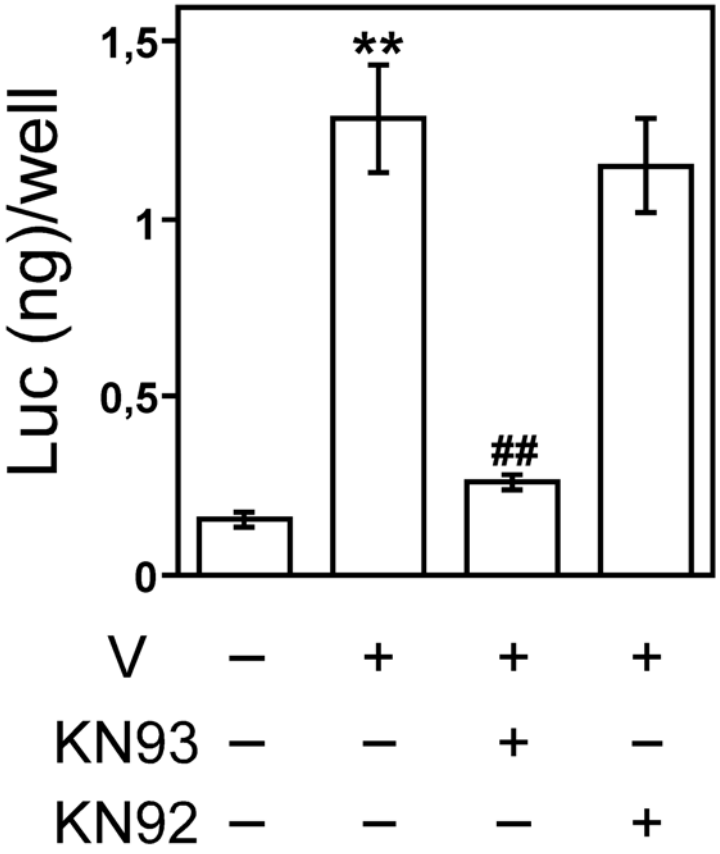


Figure 21. Treatment of the H9c2T1 cardiomyoblasts with the CaMK inhibitor KN93. Vasopressin (1 μM) stimulation led to an 8.3-fold increase in luciferase level, which was decreased by 79.5% when the cells were additionally pretreated with KN93 (10 μM). The inactive analogue KN92 (10 μM) had no influence. This suggests that the CaMKs play a pronounced role in the activation of the hALC-1 promoter. V, vasopressin; +,

treated; -, untreated. Bars are the means ± SEM of triplicate experiments. Significance analysis was performed using ANOVA and Bonferroni as a post test. ** $p<0.01$ (compared versus V-, KN93- KN92 -);## $p<0.01$ (compared versus V+, KN93-, KN92-).

#### 4.5.3.2 Localization of CaMK forms

Having demonstrated a role of CaMKs in hALC-1 promoter regulation, the involvement of the different forms of the CaMK family was to be analyzed. Therefore, H9c2T1 cardiomyoblasts were stimulated with vasopressin (1 μM). Subsequently, immunofluorescence staining was performed with antibodies raised against CaMKIIδ and CaMKIV. Figure 22a shows that CaMKIIδ is located exclusively in the cytoplasm and remained there even after vasopressin stimulation. In contrast, CaMKIV is located both in the cytoplasm and in the nucleus. Interestingly, a pronounced nuclear accumulation of CaMKIV upon vasopressin stimulation of H9c2T1 cardiomyoblasts was observed (Fig. 22b).

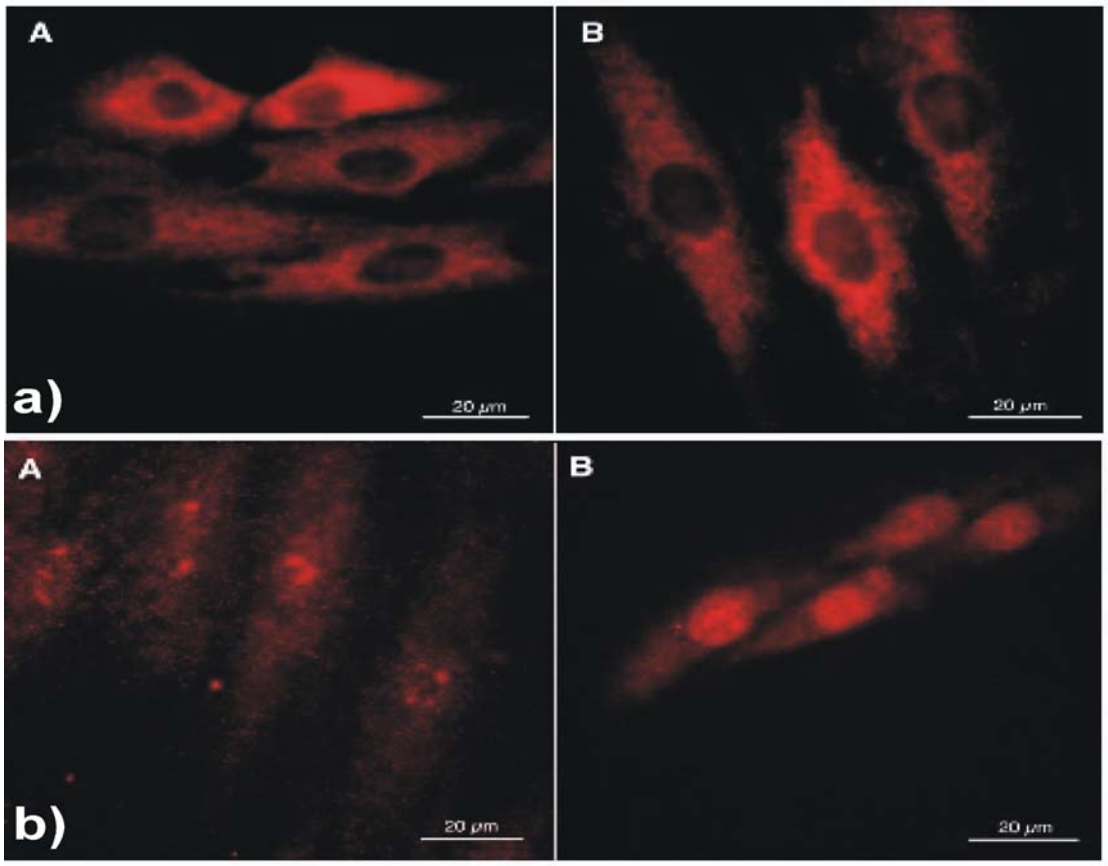


Figure 22. Immunofluorescence and localization of CaMK forms. a): Immunofluorescence of H9c2T1 cardiomyoblasts with an antibody raised against CaMKIIδ, visualized by Alexa 594. The staining pattern shows that CaMKIIδ is located in the cytoplasm before (A) and after (B) vasopressin (1 μM) treatment. b): Immunofluorescence of H9c2T1 cardiomyoblasts using an antibody raised against CaMKIV, visualized by Cy3. CaMKIV is located in the cytoplasm as well as in the nucleus in unstimulated (without vasopressin) (A) and vasopressin (1 μM)-stimulated cells (B), but there was a pronounced nuclear accumulation of CaMKIV in stimulated cells (B).

### 4.6 Analysis of hALC-1 promoter activity in a second stably transfected cardiomyoblast line (H9c2T2)

Stimulation of H9c2T2 cardiomyoblasts with vasopressin led to a significant activation of the hALC-1 promoter (Fig. 23). Moreover, inhibition of CaMKs with KN93 significantly reduced the activity of the hALC-1 promoter, shown by a decreased luciferase signal (Fig. 23). These experiments demonstrate that the results obtained with the H9c2T1 cardiomyoblast line could be reproduced in a second stable cell line (H9c2T2).

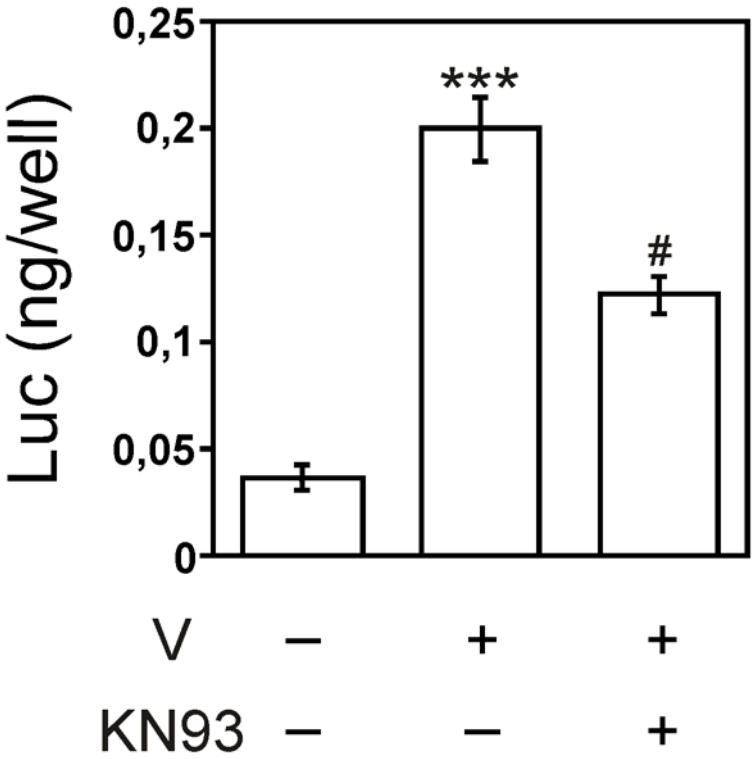


Figure 23. Treatment of H9c2T2 cardiomyoblasts with vasopressin and the CaMK inhibitor KN93. Stimulation of H9c2T2 cardiomyoblasts with vasopressin (1μM) caused an increase in hALC-1 promoter activity shown by a 5.5-fold rise of the luciferase level. When H9c2T2 cardiomyoblasts were additionally pretreated with the inhibitor KN93 (10 μM), a significant reduction in hALC-1 promoter activity was observed. V, vasopressin; +, treated; -, untreated. Bars are the means ± SEM of triplicate experiments. Significance analysis was performed using ANOVA and Bonferroni as a post test. *** $p<0.001$ (compared versus V-, KN93-); # $p<0.05$ (compared versus V+, KN93-).

### 4.7 Analysis of consensus binding sites in the hALC-1 promoter

Using the Genomatix Matinspector software, the sequence of the hALC-1 promoter (cf. Appendix 1) was analyzed for the detection of consensus binding sites for transcription factors. Table 3 lists a number of the sites, which have been implicated in the development of hypertrophy and are therefore important for the analysis of the described cell model and the interpretation of the observed effects.

| **Transcription factor** | **Consensus binding site** | **Location in hALC-1 promoter sequence** |
|---|---|---|
| NFAT | 5'-GGAAA-3' | -1679 to -1689 bp |
| GATA-4 | 5'-(A/T) GATA (A/G)-3' | -1484 to -1496 bp |
| MEF2 | 5'-CT (A/T) (A/T) AAATAG-3' | -1595 to -1617 bp |
| CREB | 5'-TGACGTCA-3' | -1558 to -1578 bp |
| AP-1 | 5'-TGA (C/G) TCA-3' | -607 to -617 bp |

Table 3. Selected list of potential transcription factor binding sites in the hALC-1 promoter.