# 5. DISCUSSION

The main finding of this work is that $Ca^{2+}$-calmodulin-dependent signaling pathways play a dominant role in the regulation of human ALC-1 expression upon hypertrophic stimulation.

The human ALC-1 is part of the cardiac molecular motor. Even though the normal adult ventricle of the human heart does not express ALC-1 (Fallot 1888), it can be found in the overloaded hypertrophied heart ventricle (Auckland et al. 1986; Schaub et al. 1984; Ritter et al. 1999b). Transgenic overexpression of hALC-1 led to improved cardiac contractility (Abdelaziz et al. 2004; Fewell et al. 1998), showing that hALC-1 plays an important role in the regulation of heart function. Despite the significance of ALC-1 for myosin function and the molecular adaptation of the human heart to compensate an increased work demand, only little information on the regulation of the hALC-1 gene expression on the promoter level is available.

To investigate the intracellular pathways which control human ALC-1 promoter activity and thereby regulate ALC-1 expression under hypertrophic conditions, a cell model was established. A reporter gene construct was generated consisting of the hALC-1 promoter cloned in front of the luciferase reporter gene and a selection marker. After verification of the reporter gene construct by DNA sequencing and restriction enzyme digestion, it was used for the stable transfection of H9c2 cardiomyoblasts. Selection with the antibiotic G418 and subcloning led to the stable cell lines H9c2T1 and H9c2T2.

The H9c2 cell line is derived from an embryonic rat heart. These cardiomyoblasts possess elements and properties of signaling pathways of adult cardiomyocytes (Hescheler et al. 1991; Hoch et al. 1998). H9c2 cardiomyoblasts also possess an intact $V_{1a}/G_q$-receptor pathway (Chen and Chen 1999) and have been used as a model for vasopressin-induced hypertrophy (Brostrom et al. 2000). Besides its role in H9c2 cells, studies have also implicated a role for vasopressin in cardiomyocytes. It has been shown that vasopressin results in a $V_{1a}$ receptor-dependent increase in protein in cardiomyocytes (Xu et al. 1999; Nakamura et al. 2000) as well as in stimulation of the 42/44 kDa mitogen-activated protein kinases (Aharonovitz et al. 1998). Also, in a model

of spontaneously hypertensive rats, $V_{1a}$ receptor activation was associated with left ventricular hypertrophy (Bird et al. 2001). These findings support a possible involvement of vasopressin in cardiomyocyte hypertrophy. Thus, vasopressin-induced hypertrophy in H9c2 cardiomyoblasts seemed to be an appropriate model for the investigations described in this study.

The integration of foreign DNA into the chromosome of host cells is not a highly frequent event (Ausubel et al. 2003). Also, transfection efficiency varies greatly among different cell types and transfection methods. Therefore, before stably transfecting the H9c2 cardiomyoblasts, different transfection methods and parameters were tried out and optimized. The calcium phosphate precipitation method was found to be most suitable in order to achieve a highly efficient transfection.

When the H9c2 cardiomyoblasts were stably transfected, the reporter gene construct randomly integrated into the host genome. Theoretically, the host DNA could have unspecific effects (integration site-effects) on the reporter gene construct and thereby influence the analysis of hALC-1 promoter activity. In order to exclude unspecific effects, a second stably transfected cell line was generated (H9c2T2).

Amplification of DNA from H9c2T1 and H9c2 cardiomyoblasts by specific primers demonstrated the presence of the reporter gene construct in the stably transfected cells as opposed to the wild type cells. Moreover, neither the morphology nor the proliferation kinetics were altered after the stable insertion of the reporter gene construct into the genome of the H9c2 cardiomyoblasts, which was demonstrated by microscopic inspection and the analysis and comparison of the proliferation curves of the stably transfected and wild type cells, respectively. This suggests that the H9c2T1 cardiomyoblasts resemble the wild type H9c2 cardiomyoblasts to a high degree.

As a read-out system, a luciferase asssay was established. Programming of a luminometer with specific parameters allowed the analysis of luminescence. Using the luminescence of a recombinant protein, a calibration curve for the calculation of the amount of luciferase was obtained. Expression levels of the luciferase reporter gene then provided a selective measure of human ALC-1 promoter activity.

I found well-detectable basal luciferase expression levels in unstimulated cardiomyoblasts, demonstrating that H9c2T1 cardiomyoblasts contain the relevant activated transcription factors involved in hALC-1 promoter regulation. Likewise, well-detectable amounts of ALC-1 mRNA could be detected in the normal human ventricle (Ritter et al. 1999b), demonstrating basal activity of the ALC-1 promoter, albeit the ALC-1 protein is not expressed in the normal human ventricle (Fallot 1888; Ritter et al. 1999b).

Vasopressin-induced hypertrophy was performed under serum-free conditions. Brostrom et al. (2000) have shown that the majority of H9c2 cardiomyoblasts is in $G_o$ after 24 hours of vasopressin stimulation in serum-free medium, i.e. not different from untreated cells. Therefore, vasopressin does not promote entry into the cell cycle (Brostrom et al. 2000), a prerequiste for the development of cellular hypertrophy. However, H9c2 cardiomyoblasts can differentiate into myotubes at confluency and under conditions of serum starvation. Therefore, a cell density was chosen, which allowed serum-free cultivation of the cells and minimized risk of myotube differentiation. In addition, microscopic inspection of the cells by two independent observers ensured the absence of myotubes during the experiments.

Stimulation of the H9c2 cardiomyoblasts with vasopressin led to activation of the hALC-1 promoter demonstrated by a significant rise in the luciferase level (in comparison to basal levels). Brostom et al. (2000) have shown that treatment of H9c2 cardiomyoblasts with vasopressin results in hypertrophy of the cardiomyoblasts, demonstrated by an increase in overall protein content. Likewise, the amount of protein rose 1.7-fold in vasopressin-treated H9c2T1 cardiomyoblasts compared with untreated H9c2T1 cardiomyoblasts. This rise is comparable with the increase in protein content shown by Brostrom et al. This effect on the human promoter seems to be species-specific, since vasopressin stimulation of H9c2T1 cardiomyoblasts did not activate the expression of the endogenous rat ALC-1 gene. In fact, cardiac hypertrophy of the rat is characterized by a shift of myosin heavy chain gene expression rather than a change of essential myosin light chain gene expression as seen in humans (Ritter et al. 1999a). Although vasopressin-induced hypertrophy is an established model (Brostrom et al. 2000), its effects on transcription of a single gene has not been elucidated so far. Therefore, I present for the first time direct evidence that vasopressin-induced hypertrophy of

cardiomyoblasts is transmitted as far as the nucleus and culminates in alteration of expression of a specific gene.

Vasopressin stimulation activates the $V_{1a}$ receptor/$G_q$-protein/PLC pathway and leads to phasic and tonic increases of intracellular free $Ca^{2+}$ and to the activation of PKC and p42 MAPK (Chen and Chen 1999). Also, stimulation of the $V_{1a}$ receptor/$G_q$-protein signaling pathway led to a hypertrophic response in primary cardiomyocytes (Tahara et al. 1998). Thus, many signaling pathways regulating expression of genes involved in the hypertrophic process become activated, in particular $Ca^{2+}$-dependent as well as MAPK-pathways (Chen and Chen 1999). Stimulation of the G-protein-coupled $V_{1a}$ receptor of H9c2T1 cardiomyoblasts, therefore, also provides the opportunity to selectively dissect the most relevant hypertrophic pathways involved in human ALC-1 promoter regulation.

$Ca^{2+}$ and $Ca^{2+}$-dependent pathways have been implicated in the development of heart hypertrophy (Passier et al. 2000). Nevertheless, demonstration of the different $Ca^{2+}$ sources involved is as yet controversial. In H9c2 cardiomyoblasts, fluorometer tracings of intracellular $Ca^{2+}$ after vasopressin stimulation have demonstrated a biphasic change of $Ca^{2+}$ levels: a peak (phasic response) followed by a lower $Ca^{2+}$ plateau (tonic response). It has also been shown that extracellular $Ca^{2+}$ is responsible for the tonic phase whereas intracellular $Ca^{2+}$ defines the phasic peak (Chen and Chen 1999). Ionomycin is an ionophore that leads to a rise in intracellular free $Ca^{2+}$ 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). Treatment of the stably transfected H9c2T1 cardiomyoblasts with this ionophore resulted in a significant rise of the luciferase level. Whereas this experiment indicated an activation of the hALC-1 promoter caused by a general increase in intracellular free $Ca^{2+}$, it did not clarify whether extra- or intracellular $Ca^{2+}$ sources were responsible for this. A subsequent experiment showed that elimination of extracellular $Ca^{2+}$ from the culture medium and thus elimination of the $Ca^{2+}$ plateau still resulted in the full vasopressin effect on hALC-1 promoter activity. I therefore suggest that phasic rather than sustained tonically enhanced intracellular $Ca^{2+}$ levels activate those $Ca^{2+}$-calmodulin-dependent pathways which regulate the expression of the hALC-1 gene.

The PKC is a serine/threonine kinase, which can phosphorylate transcription factors and thereby influence the transcription of target genes (Clerk et al. 1994). Upon stimulation of G-protein-coupled $V_{1a}$ receptor, activated PKC mobilizes the p42 MAPkinase (ERK2) pathway in H9c2 cardiomyoblasts (Chen and Chen 1999). Transgenic overexpression of a MAPKK that activates ERK1/2 has been shown to result in a hypertrophic phenotype of the heart (Bueno et al. 2000). ERK1/2 is believed to phosphorylate nuclear transcription factors, thus increasing the transcription rate of cardiac genes (Davis 1993). Also, different PKC isoforms have been implicated in the development of heart hypetrophy. It has been shown that the PKC subtypes $\delta$ and $\varepsilon$ and not the PKC$\alpha$ were translocated to the membrane upon hypertrophic stimulation and that this activates the MAPK pathway in neonatal cardiomyocytes (Clerk et al. 1994). In order to obtain information on the involvement of the PKC isoforms and, indirectly, on the MAPK pathway in the vasopressin-stimulated H9c2T1 cardiomyoblasts, the PKC inhibitor bisindolylmaleimide (BIM) was used. This inhibitor is highly selective for PKC. It also allows the differentiation between the various PKC isoforms, since lower concentrations of BIM lead to the inhibition of subtypes $\alpha$ and $\beta$, whereas inhibition of subtypes $\delta$ and $\varepsilon$ is only achieved at higher concentrations (Martiny-Baron et al. 1993; Budworth and Gescher 1995; Toullec et al. 1991). BIM revealed only a small and statistically non-significant inhibitory effect on human ALC-1 promoter activity. This suggests that the different PKC isoforms are not involved in the regulation of human ALC-1 promoter activity in H9c2T1 cardiomyoblasts upon vasopressin stimulation. It also indicates that other signaling pathways play a dominant role in human ALC-1 promotor activity regulation.

Activation of the protein phosphatase calcineurin by the $Ca^{2+}$-calmodulin complex and subsequent dephosphorylation and nuclear translocation of transcription factors of the NFAT family is a major mechanism of gene transcription activation in the course of the cardiac hypertrophy process (Molkentin et al. 1998; Ritter et al. 2002). I could demonstrate that the same pathway is active in the H9c2T1 cardiomyoblast cell line and that it increased human ALC-1 promoter activity. Analysis of localization of NFAT was done using an immunofluorescence labelled antibody against NFAT3, since this family member has been reported to be involved in human heart hypertrophy (Molkentin et al. 1998). Immunofluorescence analysis revealed significant (approximately 93%) nuclear

translocation of NFAT3 upon vasopressin stimulation. Since the vasopressin-induced nuclear translocation of NFAT3 is very prominent, further experiments were carried out in order to confirm the specificity of this effect. NFAT3 immunofluorescence staining of untreated and vasopressin-treated H9c2T1 cardiomyoblasts was almost completely absent in the presence of a blocking peptide. In order to eliminate the possibility that fluorescence from, e.g., the nuclear membrane is a source of false positive signals from the nuclei, confocal microscopy was carried out. It confirmed that only the nuclei are the source of the observed fluorescence pattern in vasopressin-treated H9c2T1 cardiomyoblasts. Cyclosporin A (CspA) is a potent calcineurin inhibitor (Leinwand 2001; Shaw et al. 1995). CspA partially but significantly attenuated the activation level of the human ALC-1 promoter of vasopressin-stimulated H9c2T1 cardiomyoblasts. Therefore, the involvement of the calcineurin-NFAT axis was confirmed on another level. Whether translocated NFAT directly activates the human ALC-1 promoter and/or affects promoter activity via integrated action with other transcription factors needs to be elucidated. NFAT3 has been demonstrated to be a co-factor for the cardiac zinc-finger-transcription factor GATA-4, which plays an important role in the cardiac hypertrophy process (Molkentin and Olson 1997). For direct activation, the human ALC-1 promoter sequence (Rotter et al. 1991) contains several NFAT as well as GATA-4 consensus binding sites (e.g. at -1679 bp to -1689 bp and -1484 bp to -1496 bp, respectively). Moreover, the significance of the calcineurin/NFAT pathway for human ALC-1 promoter activity is in line with clinical investigations: patients with hypertrophic obstructive cardiomyopathy revealed significantly elevated calcineurin activity (Ritter et al. 2002) and expressed large amounts of ALC-1 (Ritter et al. 1999b).

The multifunctional CaMKs have been shown to play a role in heart hypertrophy (Maier and Bers 2002). Therefore, I investigated their involvement by using the CaMK inhibitor KN93, which blocks activity of all CaMKs (Hook and Means 2001). KN92 is an inactive analogue of KN93 and is an adequate negative control in order to exclude unspecific side effects of KN93 (Tombes et al. 1995). In contrast to the calcineurin pathway, which only partially activated the human ALC-1 promoter, I found an almost complete decline of promotor activity to basal levels upon CaMK inhibition of vasopressin-stimulated H9c2T1 cardiomyoblasts. Again, this shows that $Ca^{2+}$-calmodulin pathways have an important impact on human ALC-1 promoter regulation. In the next step, the presence and localization of CaMKII$\delta$ and CaMKIV were analyzed, since these specific CaMKs

have been demonstrated to be involved in cardiac hypertrophy processes (Maier and Bers 2002). In fact, a nuclear localization of CaMKIIδ was found to be important for its activation of cardiac gene expression (Ramirez et al. 1997). Also, CaMKIV is mainly found in the nucleus (Jensen et al. 1991). Therefore, a nuclear CaMK localization is more likely to confer the transcriptional effects in the H9c2T1 cardiomyoblasts after vasopressin stimulation. H9c2T1 cardiomyoblasts expressed cytoplasmic forms of the CaMKIIδ, which is in line with another publication (Hoch et al. 1998). This localization pattern remained unchanged upon vasopressin stimulation. CaMKIV, however, could be localized in both the cytoplasm and in the nucleus in unstimulated H9c2T1 cardiomyoblasts. Upon vasopressin stimulation, CaMKIV accumulated in the nucleus. This vasopressin-induced relative nuclear accumulation of CaMKIV is interesting, since CaMKIV confers hypertrophic responses upon phosphorylation and subsequent nuclear export of HDAC, leading to the release of active MEF2 isoforms from the HDAC/MEF2 complex (Passier et al. 2000). Furthermore, the hypertrophic response upon stimulation of G-protein-coupled receptor of primary neonatal cardiomyocytes by phenylephrine activated MEF2, a process, which was closely associated with phosphorylation of HDACs by CaMKIV (Lu et al. 2000). Several corresponding consensus binding sites for MEF2 exist in the human ALC-1 promoter sequence (e.g. at –1595 bp to -1617b p). Alternatively, CaMKIV phosphorylates CREB, a ubiquitous transcription factor, which is then co-activated by, e.g., CREB-binding protein (CBP) (Sheng et al. 1991). Phosphorylation of CREB by CaMKIV occurs at the same site (Ser133) as PKA-mediated phosphorylation (Sheng et al. 1991), which markedly stimulates cAMP-response element (CRE)-mediated transcription. A CREB binding site in the human ALC-1 promoter (-1558 bp to -1578 bp) exists. CaMKII, however, additionally phosphorylates CREB at S142, thus negatively regulating CREB activity (Sun et al. 1994). Likewise, ATF-1 is stimulated only by CaMKIV phosphorylation at Ser 63, while CaMKII was ineffective due to the phosphorylation on a second site at ATF-1 (Sun et al. 1996). Hence, in the H9c2T1 cardiomyoblast cells, the stimulation of human ALC-1 promoter activity by CaMKs may well be mediated by CaMKIV rather than CaMKII. It has been hypothesized that CaMKII may functionally substitute for CaMKIV (Chien 2000). Therefore, CaMKII with its isoform δ3 may be the relevant nuclear CaMK in the human heart.

Interestingly, the demonstrated inhibitor experiments suggest a link between the calcineurin and the CaMK pathway. Even though the performed immunofluorescence studies showed more than 90% NFAT translocation in response to vasopressin, calcineurin inhibition only led to a decrease of roughly 52% in the vasopressin-induced activation of the hALC-1 promoter. However, CaMK inhibition revealed a reduction of approximately 80% of the promoter activity. This implies that the CaMK pathway is more essential and that it could influence calcineurin- and NFAT-dependent signaling. Even though KN93 had only a small influence on NFAT activation in a study using vascular smooth muscle cells (Afroze et al. 2003), several possibilities for cross-talk exist. First, upstream of NFAT binding to the respective promoter region, calcineurin could be activated by CaMKs. However, a direct phosphorylation of calcineurin by CaMKs such as controversially discussed for ERK kinase (Aramburu et al. 2004) has not been demonstrated yet. Calcipressins comprise a family of calcineurin inhibitors. Their complex phosphorylation pattern is involved in the negative and positive regulation of calcineurin activity. It cannot be ruled out that CaMKs could in turn influence the phosphorylation state of calcipressins and thereby act as a modulator of calcineurin-dependent pathways. Second, an interaction of several transcription factors coupled differentially to CaMK and calcineurin signaling could be needed for full activation of the hALC-1 promoter. It is known that NFAT cooperates with other transcription factors in order to alter gene expression (Molkentin et al. 1998; Rooney et al. 1995). It has been demonstrated that MEF2 can be activated by CaMKIV (Passier et al. 2000) and that MEF2 can bind to and synergize with NFAT (Im and Rao 2004). One other possible cofactor of NFAT is AP-1, which is a dimeric complex that comprises members of the ubiquitous transcription factor families Fos and Jun. AP-1 has been implicated in the regulation of a number of genes involved in cell growth and differentiation. Binding of the NFAT: AP-1 complex to composite DNA sites is well-known (Im and Rao 2004; Macián et al. 2001). An AP-1 site in the hALC-1 promoter is present (-607 bp to -617 bp). Interestingly, AP-1 has been reported to be activated by CaMKIV in T- lymphocytes (Ho et al. 1996). Furthermore, as described above, recent data indicates that phosphorylation of HDACs by CaMKs results in activation of MEF2 and that this collaboration may be part of hypertrophic signaling (Aramburu et al. 2004; Im and Rao 2004). I suggest that the findings shown here could be explained by the following mode of action. As already described, NFAT cooperates with other transcription factors like AP-1 and MEF2. Possibly, regulation of the hALC-1 promoter by NFAT is dependent on

that cooperation. In that case, NFAT alone could not activate the promoter. But under inhibition of the calcineurin-NFAT pathway, AP-1 and MEF2 could still be active through the influence of CaMKIV signaling and lead to an activation of the hALC-1 promoter. I have shown that in response to vasopressin, a relative translocation of CaMKIV to the nucleus takes place, which indicates a role for this CaMK in the hypertrophic signaling influencing hALC-1 promoter activity. On the other hand, when the CaMK pathway is blocked, neither NFAT nor AP-1 nor MEF2 can activate the promoter, which leads to a much stronger reduction of the signal. Therefore, I suggest that a complex interplay of the above-mentioned transcription factors may produce that pattern of inhibition of hALC-1 promoter activation observed.

Stimulation with vasopressin and inhibition of CaMKs with KN93 in the second stably transfected cell line H9c2T2 led to results comparable to those obtained with the H9c2T1 cardiomyoblasts. This demonstrates that the hALC-1-promoter-reporter gene construct is regulated in the same way in both stably transfected cell lines. It also suggests that the observed effects on hALC-1 promoter activity in the H9c2T1 cardiomyoblasts are not of an unspecific nature.

In summary, $Ca^{2+}$-calmodulin-dependent processes rather than PKC-activated hypertrophic signaling pathways, which are involved in the development of cardiac hypertrophy, regulate the activity of the human ALC-1 promoter via the activation of calcineurin and CaMKs, most probably CaMKIV (overview see Fig. 24).

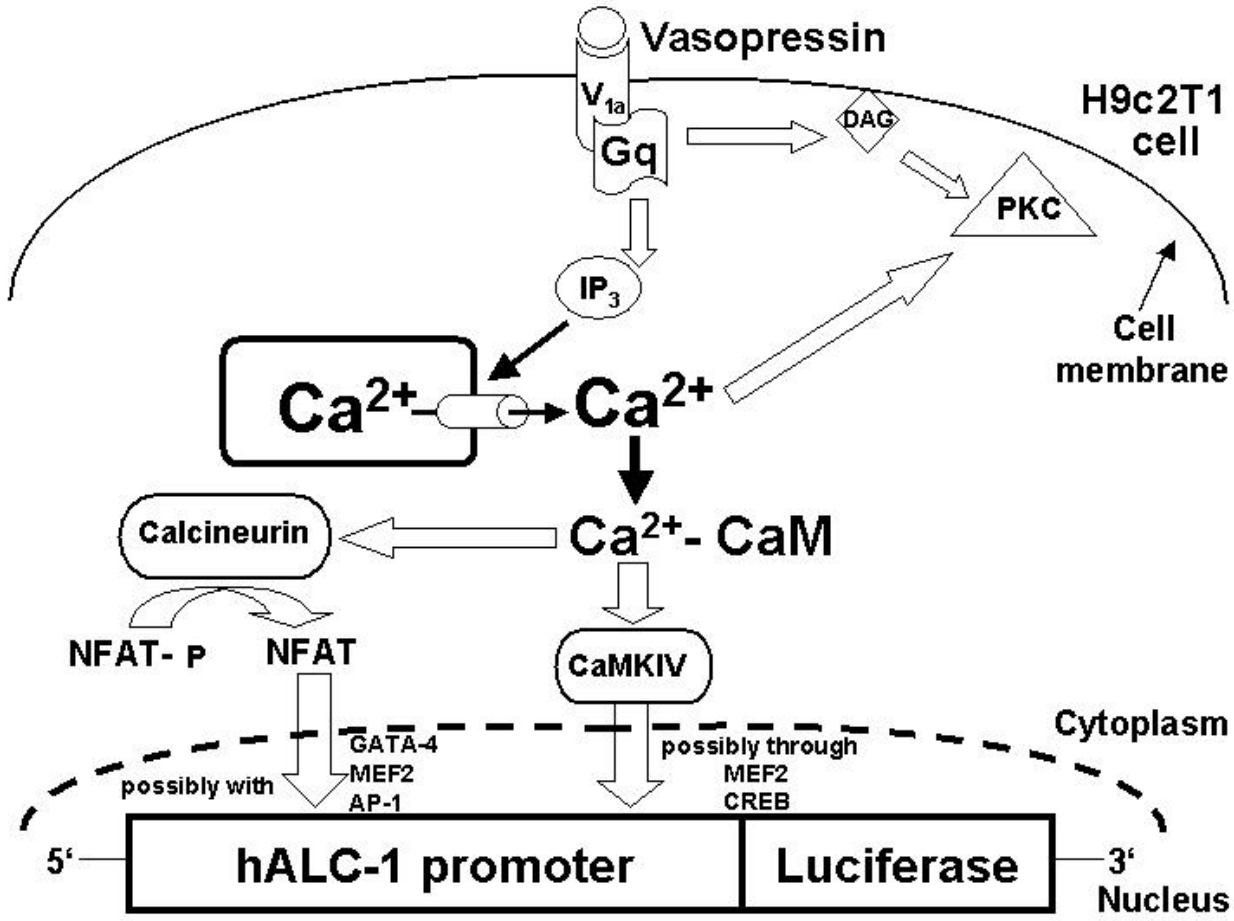


Figure 24. Overview of the proposed signaling cascade in H9c2T1 cardiomyoblasts upon vasopressin stimulation. Vasopressin leads to hypertrophy of H9c2T1 cardiomyoblasts and significant activation of the hALC-1 promoter by binding to the $V_{1a}$ receptor in the plasma membrane and activation of several signaling pathways. The receptor hormone complex activates an associated $G_q$-protein, which leads to the activation of membrane-bound phospholipase. This results in subsequent hydrolysis of phosphatidylinositol 4,5-bisphosphate in the membrane, which in turn catalyzes the production of the second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 diffuses from the plasma membrane to the endoplasmic reticulum (ER) and causes $Ca^{2+}$ channels within the ER to open. Sequestered $Ca^{2+}$ is thus released into the cytosol leading to a rise in intracellular free $Ca^{2+}$. Extracellular $Ca^{2+}$ sources are not involved in activation of the hALC-1 promoter. DAG cooperates with $Ca^{2+}$ in activating protein kinase C (PKC). However, in H9c2T1 cardiomyoblasts, the different PKC isoforms do not influence hALC-1 promoter regulation under hypertrophic conditions. $Ca^{2+}$ binds to calmodulin (CaM) leading to its activation. This

$Ca^{2+}$-calmodulin complex then activates calcineurin, which dephosphorylates the nuclear factor of activated T cells (NFAT), leading to its translocation into the nucleus. NFAT acts as a transcription factor in the activation of the hALC-1 promoter either by itself or possibly in combination with other factors (e.g. GATA-4, MEF2 and AP-1). The $Ca^{2+}$-calmodulin complex also binds to $Ca^{2+}$-calmodulin-dependent protein kinases (CaMKs). CaMKIV accumulates in the nucleus and influences ALC-1 promoter activation via several pathways. First, activated nuclear CaMKIV may result in phosphorylation of histone deacetylases and their subsequent nuclear export and release of active MEF2. Second, CaMKIV phosphorylates CREB leading to its activation. Both MEF2 and CREB then bind to their corresponding sites in the hALC-1 promoter and thus lead to the activation of the hALC-1 promoter.