1. INTRODUCTION

Homeostasis is the property of a living organism to regulate its internal environment to maintain a stable, constant condition by means of multiple dynamic equilibrium adjustments, controlled by interrelated regulation mechanisms (Cannon 1932). Homeostasis of the heart can be achieved by acute as well as chronic adaptational mechanisms. Thus, acute changes of the cardiac environment, e.g., by increased ventricular filling volumes can be balanced by the Frank Starling mechanism or elevated sympathetic tone. Chronic cardiac overload can be balanced by hypertrophic responses.

1.1 Cardiac hypertrophy

Hypertrophy is defined as the growth of a tissue or an organ through an increase in cell size rather than an increase in cell number (i.e. hyperplasia). Different environmental signals cause distinct architectural changes in the heart. Thus, concentric hypertrophy, characterized by a decrease in cavity volume and eccentric hypertrophy with dilatation of the heart, takes place upon enhanced pressure-overload and volume-overload, respectively (Poche 1996). Moreover, concentric hypertrophy is characterized by parallel addition of sarcomeres and lateral growth of single cardiomyocytes, whereas addition of sarcomeres in series and longitudinal cell growth is the hallmark of eccentric hypertrophy (Dorn II et al. 2003). The hypertrophic remodeling process of the heart also includes capillary formation, fibroblast proliferation and stromal tissue disposition (Tarone and Lembo 2003). At the microscopic level, besides the normal set of diploid chromosomes, the cardiomyocyte nucleus can undergo polyploidy (Van der Laarse et al. 1998) leading to tetraploid, octoploid or even higher sets of chromosomes (Lips et al. 2003). Characteristically, a reinduction of the fetal gene program, enhanced protein synthesis and an increase in sarcomere assembly take place (Fiedler and Wollert 2004; Schwartz et al. 1986; Dorn II et al. 2003)

1.1.1 Mechanosensors couple wall stress to intracellular signals

According to the law of Laplace, the wall stress “K” of the heart is proportional to the pressure development “p” and the radius “r” of the ventricle (Equ. 1) (Schmidt and Thews 1995).

$$K = p \frac{r}{2d}$$

K= wall stress
p= ventricular pressure
r= radius of ventricle
d= wall thickness

Equation 1

Thus, any increase in the enddiastolic ventricular filling volume, which raises “r”, or increased afterload, which is compensated by a rise in the pressure development “p”, increases the wall stress. Since wall stress is inversely proportional to the ventricular wall thickness “d”, hypertrophy of the ventricular wall which increases “d” normalizes wall stress according to Equation 1. Different mechanotransduction-sensors have been suggested to couple wall stress to intracellular signals, inducing the hypertrophic response (Ruwhof and Van der Laarse 2000) (overview see Fig. 1).

Mechanical stress of the cardiomyocyte leads to deformation of the sarcolemma. This, in turn, results in conformational changes and subsequent activation of transmembrane proteins such as integrins, ion exchangers such as the Na^+/H^+ exchanger and specific stretch-activated channels (Ruwhof and Van der Laarse 2000) (Fig. 1).

Integrins are cell adhesion receptors and act as cellular mechanoreceptors (Ross et al. 2004). Recently, it has been shown that their cytoplasmic domain is linked to intracellular signaling molecules such as α-actinin and focal adhesion kinase (FAK) (Hynes 1992; Lewis and Schwartz 1995). Mechanical stimulation of cultured cardiomyocytes simulating hemodynamic loading of the intact heart activated a number of integrin-dependent intracellular signaling pathways (Sadoshima et al. 1993). Synergy between growth factor and integrin-induced hypertrophy has been described. Integrins

were shown to use FAK and Ras to transduce hypertrophic signals towards p38 mitogen-activated protein (MAP) kinase (MAPK) (Aikawa et al. 2002).

Mechanical stress-induced hypertrophy of cardiomyocytes *in vitro* (Yamazaki et al. 1998) and *in vivo* (Takewaki et al. 1995) was associated with an enhanced activity of the cardiac Na^+/H^+-exchanger (NHE). The elevated intracellular sodium (Na^+) concentration subsequently led to a rise in intracellular Ca^{2+} via the Na^+/Ca^{2+}-exchanger (NCX) (Cingolani and Camilion de Hurtado 2002). Furthermore, inhibition of the NHE by cariporide led to a reduction of cardiac hypertrophy in rats after myocardial infarction (Kusomoto et al. 2001).

Stretch-activated channels (SACs) are mechanosensitive ion channels which allow passage of ions such as Na^+, K^+ and Ca^{2+} (Hu and Sachs 1997). Activation of these channels has been suggested to be a transduction mechanism between mechanical stress and heart hypertrophy (Ruknudin et al. 1993).

Furthermore, sensors of mechanical stress cause a rise in intracellular Ca^{2+}, leading to the stimulation of signaling cascades such as calcineurin-, calcium-calmodulin-dependent protein kinases (CaMKs)-, protein kinase C (PKC)- and MAPK-dependent pathways, all of which are involved in cardiac hypertrophy (Frey et al. 2000) (Fig. 1).

Enhanced wall stress also leads to the release of a variety of autocrine and paracrine factors (Fig. 1). Mechanical overload leads to the release of inflammatory cytokines such as interleukin 1 (IL-1) and interleukin 6 (IL-6), tumor necrosis factors α (TNF-α) and insulin-like growth factor 1 (IGF-1) (Tarone and Lembo 2003). IL-1 and IL-6 have been shown to lead to concentric heart hypertrophy (Isoda et al. 2001; Hirota et al. 1999). TNF-α overexpression results in dilated cardiomyopathy (Kubota et al. 1997). IGF-1 is important for the protection of cardiomyocytes from hypertrophy-associated apoptosis (Li et al. 1997). However, ventricular hypertrophy due to hemodynamic overload is not affected by changes in IGF-1 levels (Lembo et al. 1996).

In addition, G-protein-coupled receptors (GPCRs) are involved in the regulation of cardiac function and adaptation to changes in neurohumoral factors. They are coupled

to the GTP-binding proteins G_s , G_q and G_i, all of which consist of the subunits $G\alpha$ and $G\beta\gamma$. Receptor activation catalyzes the exchange of GDP for GTP and leads to the dissociation of the subunits and independent activation of intracellular signaling pathways (Pollard and Earnshaw 2004). In the heart, adrenergic (subtypes of α- and β-adrenergic receptors) and muscarinergic receptors belong to the most important GPCRs. Adrenergic signal molecules binding adrenoreceptors can cause cardiac hypertrophy (Knowlton et al. 1993; Milano et al. 1994). Specifically, in a model of cardiac pressure-overload in the rat, the amount of norepinephrine was decreased in the left ventricle. Norepinephrine reduction has been associated with an increased catecholamine turnover in the heart and an elevated cardiac sympathetic neurotransmission (Ganguly et al. 1989; Ganguly and Sherwood 1991; Siri 1988). This, in turn, has been implicated to play a role in the process of heart hypertrophy (Wendell et al. 2000).

The renin-angiotensin system, a regulator of salt and water equilibrium and consequently also of plasma volume, is also an important humoral signaling system involved in cardiac hypertrophy (Tarone and Lembo 2003). Angiotensin II (Ang II) has been demonstrated to promote hypertrophy and to contribute particularly to the hypertrophic response to mechanical overload, mediated through increased systemic levels as well as through local production in the heart (Schunkert et al. 1990). However, even though AT1α is the main Ang II receptor in the heart, its involvement in cardiac hypertrophy is not clear (Tarone and Lembo 2003; Harada et al. 1998). Basic fibroblast growth factor (bFGF) is an example of a paracrine and autocrine factor, since it is released by fibroblasts and cardiomyocytes, respectively (Tarone and Lembo 2003). bFGF has been found to be involved in pressure-overload and Ang II-induced cardiac hypertrophy (Schultz et al. 1999; Pellieux et al. 2001). These stimuli also lead to production of transforming growth factor β (TGF-β), which in turn takes part in mediating cardiomyocyte hypertrophy (Schultz Jel et al. 2002; Kuwahara 2002).

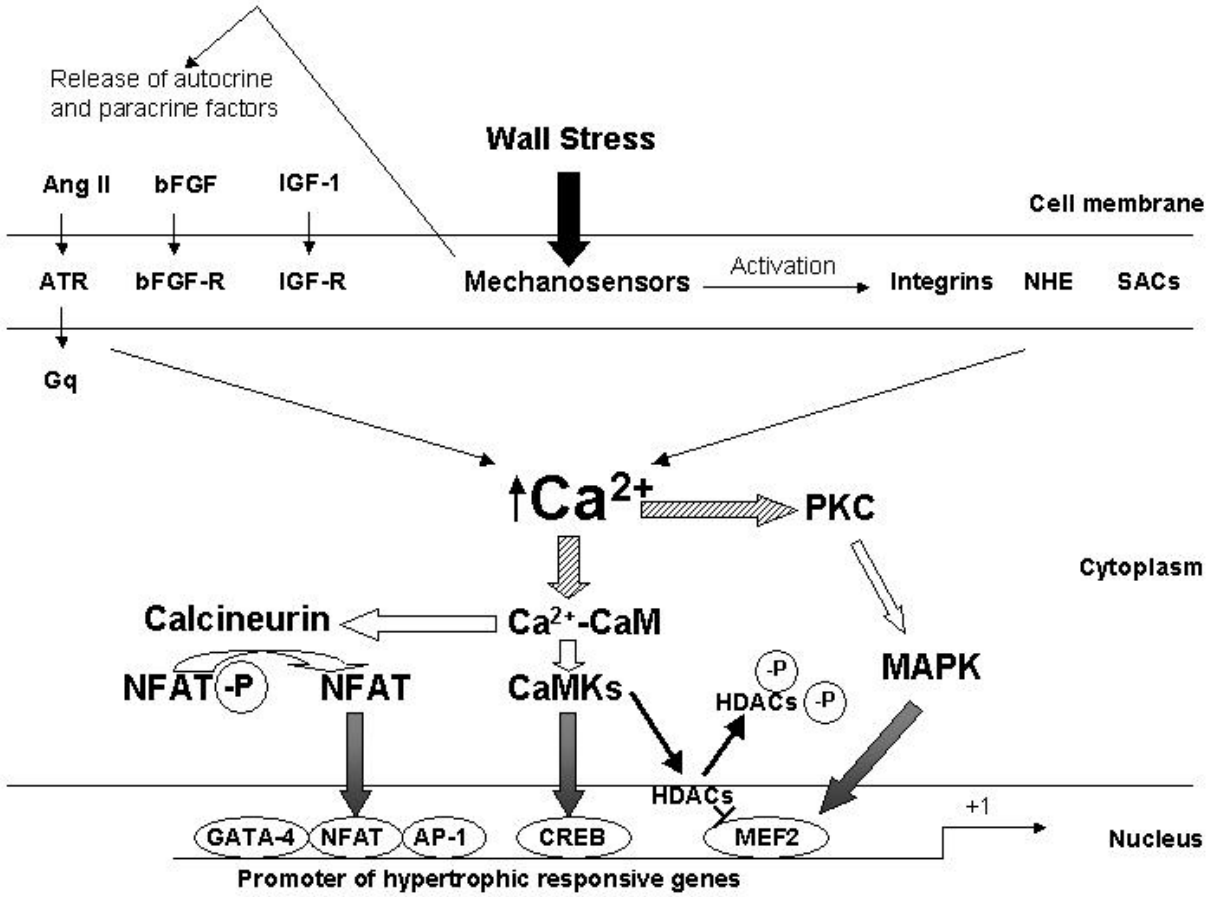

Figure 1. Overview of wall stress-induced signaling pathways in the heart. Wall stress activates a panel of signaling pathways via two parallel mechanisms. On the one hand, autocrine and paracrine factors such as angiotensin II (Ang II), basic fibroblast growth factor (bFGF) and insulin-like growth factor 1 (IGF-1) are released, which act via their respective receptors (ATR, bFGF-R and IGF-R). G-proteins of the G_q class can be involved in subsequent signaling. In addition, mechanosensors directly activate transmembrane proteins such as integrins, the Na^+/H^+ exchanger (NHE) and stretch-activated channels (SACs). Both pathways can culminate in the increase in intracellular Ca^{2+} and the subsequent activation of several signaling cascades. Ca^{2+} binds to calmodulin (CaM) and activates calcineurin, which dephosphorylates the nuclear factor of activated T cells (NFAT). NFAT then translocates into the nucleus and binds to the promoter of hypertrophic responsive genes, also in combination with other transcription factors such as GATA-4 and activator protein-1 (AP-1). Ca^{2+}-CaM additionally activates the multifunctional Ca^{2+}-calmodulin-dependent protein kinases (CaMKs). By regulating transcription factors such as the cAMP-response element-binding protein (CREB), CaMKs can influence gene expression under hypertrophic conditions. Myocyte

enhancer factor 2 (MEF2) is a transcription factor which is repressed through binding to histone deacetylases (HDACs). Phosphorylation of HDACs by CaMKs leads to their export into the cytoplasm and release of MEF2. Ca^{2+}-dependent activation of protein kinase C (PKC) and subsequent activation of the mitogen-activated protein kinase (MAPK) pathway may also result in MEF2-dependent transcription.

1.1.2 Vasopressin and cardiac hypertrophy

Arginine vasopressin (vasopressin), also called antidiuretic hormone (ADH), is a peptide hormone and is synthesized in the neuroendocrine cells of the supraoptic and paraventricular nuclei of the hypothalamus (Berne and Levy, 1993). The synthesized hormone is then stored in the nerve terminals of the neurohypophysis (posterior pituitary). The secretion of vasopressin by the posterior pituitary into the blood can be influenced by osmotic (plasma osmolality) and hemodynamic (blood volume and pressure) changes. In addition, vasopressin secretion can be influenced by atrial natriuretic peptide (ANP) (inhibits) and Ang II (stimulates) (Berne and Levy 1993). In a model of isolated rat hearts exposed to pressure-overload it has been demonstrated that the heart itself is capable of producing vasopressin and that the peptide was released into the coronary effluents (Hupf et al. 1999). Also, little hormone synthesis has been shown in endocrine tissues such as ovary, testis and endothelial cells of pulmonary, renal and mesenteric arteries (Berne and Levy 1993).

1.1.2.1 Vasopressin receptors

Vasopressin binds to specific V_{1a}-vascular, V_2-renal and V_{1b}- (or V_3-) pituitary membrane receptors, which are coupled to distinct second messengers (Thibonnier et al. 1998). V_{1a} receptors are located on vascular smooth muscle cells, hepatocytes, blood platelets, lymphocytes and monocytes, type II pneumocytes, the adrenal cortex, the brain (hippocampus, septum and amygdalae), reproductive organs, retinal epithelium, renal mesangial cells and cell lines such as A10 (Thibonnier 1992; Thibonnier et al. 1993) and H9c2 (Chen and Chen 1999). The presence of functional V_{1a} receptors on neonatal as well as adult rat cardiomyocytes has been shown (Xu et al. 1999; Fukuzawa et al. 1999). V_{1a} receptors mediate vasoconstriction, proliferation (mitogenic actions), platelet aggregation, coagulation factors release, glycogenolysis

(Thibonnier et al. 1996) and cellular hypertrophy (Brostrom et al. 2000). V_{1b}- (or V_3-) pituitary receptors are located in the anterior pituitary, where they stimulate the release of adrenocorticotropine hormone (ACTH) and β-endorphin (Thibonnier et al. 1996). V_2-renal receptors are found in the MDCK and LLC-PK_1 cell lines and in the medullary portion of the kidney, where they regulate free water and urea reabsorption via stimulation of adenylate cyclase (Thibonnier et al. 1996). Mutations of the different vasopressin receptors can lead to monogenic as well as complex human diseases. Also, overexpression of V_{1a} and V_3 receptors have been observed in a number of endocrine tumors (Thibonnier 2004).

Vasopressin binding to its corresponding receptor has been shown to induce a lateral mobility, which facilitates their interaction with G-proteins (Lutz et al. 1991). These receptor-ligand complexes are then subjected to receptor-mediated endocytosis (Jans et al. 1990). Delivery to the intracellular endosomes and subsequently to the lysosomes (degradative pathway) or to the plasma membrane (recycling pathway) follows. The source and the type of the receptor seems to determine which of the pathways is persued (Jans et al. 1999; Fishman et al. 1985).

It has been demonstrated that V_{1a} receptors are coupled to the G_q-class of G-proteins (Wange et al. 1991). Effects of the immediate transmembrane signaling of the V_{1a} receptor include activation of phospholipases A2, C and D, production of inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG), mobilization of intracellular Ca^{2+} (Exton 1990; Thibonnier et al. 1991a; Thibonnier 1992; Welsh et al. 1990), translocation and activation of protein kinase C (Gallo-Payet et al. 1991), alteration of intracellular pH (with activation of the Na^+/H^+ exchanger) (Berk et al.1987; Thibonnier 1992) and calmodulin activation (Thibonnier et al. 1987). Tyrosine phosphorylation, induction of gene expression (Thibonnier et al. 1991b; Sellmayer et al. 1991) and protein synthesis (Geisterfer and Owens 1989) belong to the secondary, nuclear signal mechanisms induced by V_{1a} receptor activation.

1.1.2.2 Physiological role of vasopressin

Regulating the excretion of water and solutes (Na^+, K^+, H^+ and urea) in the kidney is the main function of the antidiuretic hormone vasopressin. This hormone has an important

function in volume homeostasis under normal physiological conditions by continously responding to changes in plasma osmolality (Nielsen et al. 1999; Berne and Levy 1993). Thus, swelling of osmoreceptors located in the hypothalamus leads to the secretion of vasopressin. Following its release into the circulation, vasopressin binds to V_2 receptors located in the collecting duct principle cells in the kidney. This leads to a G-protein and protein kinase A (PKA)-coupled synthesis of water channels and their insertion into the apical membrane culminating in the reabsorption of free water, thereby decreasing plasma osmolality (Nielsen et al. 1999; Berne and Levy 1993). Vasopressin is also responsible for the maintenance and regulation of vascular tone via V_{1a} receptors, which are located on vascular smooth cells. Cardiopulmonary and sinoaortic baroreceptors respond to stretch and detect reductions or increases in pressure, which in turn leads to a rise or a decrease in the production and release of vasopressin (Goldsmith 1987; Berne and Levy 1993). Stimulation of the V_{1a} receptors by vasopressin leads to potent arteriole vasoconstriction resulting in significant increases in systemic vascular resistance (SVR) (Goldsmith 1987). However, at the same time vasopressin also potentiates the sinoaortic baroreceptor reflex in response to elevated SVR via the V_2 receptor (Goldsmith 1987; Ebert et al. 1986). This leads to the reduction of both heart rate and cardiac output to maintain constant blood pressure (Ebert et al. 1986; Sampey et al. 1999). Therefore, in healthy individuals under normal physiological conditions, vasopressin release increases SVR without increasing blood pressure via stimulation of both V_{1a} and V_2 receptors (Ebert et al. 1986).

1.1.2.3 Role of vasopressin in cardiomyocytes

While vascular actions of vasopressin have been established, it is still unclear whether vasopressin can exert direct action on the human heart. Nevertheless, in order to unravel the role of vasopressin in cardiomyocytes, multiple studies have been carried out in isolated cardiomyocytes of different origin.

Involvement of the V_{1a} receptor G-protein-coupled signaling pathway in cardiomyocytes has been studied intensively. By now it is well-accepted that vasopressin induces an increase in intracellular free Ca^{2+} in neonatal rat cardiomyocytes (Liu et al. 1999; Xu et al. 1999; Xu and Gopalakrishnan 1991) and in isolated perfused adult rat hearts (Fukuzawa et al. 1999). Moreover, involvement of p42/44 mitogen-activated protein

kinases (MAPKs) (Aharonovitz et al. 1998) and an increased expression of c-fos, a member of the immediate-early gene program, has been described in response to vasopressin in cardiomyocytes (Nakamura et al. 2000). Several studies have shown that vasopressin leads to a V_{1a} receptor-dependent increase in protein in cardiomyocytes (Xu et al. 1999; Nakamura et al. 2000).

Vasopressin has exerted positive as well as negative inotropic effects in a number of animal models, depending on the achieved systemic concentrations (Lee et al. 2003). In dogs, rats and guinea pig hearts, high concentrations of vasopressin led to a decrease in cardiac contractility (Khayyal et al. 1985; Walker et al. 1988; Fujisawa and Iijima 1999). However, when a vasopressin infusion within the physiological range was applied to rats, a positive inotropic effect was evoked (Walker et al. 1988). Also, cardiomyocytes showed a dose-dependent increase in contractility in response to V_{1a} receptor-mediated vasopressin stimulation (Chandrashekhar et al. 2003).

Several studies implicate a role for vasopressin in the development of cardiac hypertrophy, which is V_{1a} receptor-dependent. Vasopressin induces hypertrophy in cultured neonatal rat heart cells (Nakamura et al. 2000). Stimulation of the 42/44 kDa MAPKs by vasopressin in rat cardiomyocytes supports a role in cardiac hypertrophy (Aharonovitz et al. 1998). Also, vasopressin directly increased the rate of protein synthesis in isolated perfused adult rat hearts (Fukuzawa et al. 1999). Moreover, a significant association between V_{1a} receptor activation and left ventricular hypertrophy was found in spontaneously hypertensive rats (Bird et al. 2001). Cardiomyocyte hypertrophy may play an important part in the pathogenesis of heart hypertrophy linked to a variety of cardiovascular diseases like congestive heart failure (Tahara et al. 1998). It has been suggested that vasopressin is possibly involved in enhanced ventricular wall stress and can play a critical role in the pathophysiology and progression of heart failure in general (Goldsmith 2002). In neonatal rat cardiomyocytes, the V_{1a}/V_2 receptor antagonist conivaptan (YM-087) inhibited the vasopressin-stimulated protein synthesis. Therefore, it was suggested that this inhibitor might have beneficial effects in the treatment of cardiomyocyte hypertrophy (Tahara et al. 1998). In a model of myocardial-infarcted rats, chronic V_{1a} receptor blockade prevented heart failure. However, changes in left ventricular hypertrophy were not associated with the improvements in cardiac function (van Kerckhoven et al. 2002). Also, in post-myocardial infarction remodeled rat

hearts, V_{1a} receptors were functionally and numerically downregulated. V_{1a} receptor blockade did not attenuate ventricular remodeling in this animal model (Chandrashekhar et al. 2003).

1.1.2.4 Vasopressin induces hypertrophy in H9c2 cardiomyoblasts

The clonal H9c2 cardiomyoblast cell line was originally derived from an embryonic rat heart, which included mostly ventricular tissue, applying a modification of selective serial passaging (Kimes and Brandt 1976; Yaffe et al. 1968). The cells are large, mostly mononucleated myoblasts, which can adopt shapes such as polygonal, spindle-shaped, spherical or angular (Kimes and Brandt 1976; Hescheler et al. 1991). However, when confluency is reached, multinucleated tubular structures begin to arise by fusion of the cells. If in addition the serum concentration of the culture medium is reduced to 1%, the fusion to myotubes occurs faster and involves almost all of the myoblasts (Kimes and Brandt 1976). It was suggested that the differentiation of H9c2 cardiomyoblasts is regulated by phosphoinositide 3-kinase (PI3K) through a protein kinase B (PKB)/Akt-independent pathway (Kim et al. 1999). Since the myotubes were found to express nicotinic receptors and to produce a muscle specific creatine phosphokinase isoenzyme, the cell line was originally described to be a skeletal muscle cell line rather than a morphologically aberrant heart muscle cell line (Kimes and Brandt 1976). Structures of freshly isolated cardiomyocytes such as gap junctions, caveolae, T-tubules or myofibrils with organized sarcomeres were not observed (Hescheler et al. 1991). But since the cytoplasm is interlaced by a network of stress fibers, it was suggested that H9c2 cells are comparable to immature embryonic cardiomyocytes (Hescheler et al. 1991). On the other hand, H9c2 cardiomyoblasts also display a number of characteristics concerning electrical, hormonal and Ca^{2+} signaling found in adult cardiac cells: 1) an inward current through Ca^{2+} channels showing the characteristics of cardiac L-type currents (i.e. fast activation kinetics, sensitivity to organic Ca^{2+} channel blockers of the dihydropyridine type, stimulation by isoproterenol) (Hescheler et al. 1991) 2) the signal transducing G-proteins found in the membrane resemble those seen in striated muscle (Hescheler et al. 1991) 3) the expression of the cardiac L-type Ca^{2+} channel (Alvarez et al. 1994; Hoch et al. 1998), of the cardiac isoform of the ryanodine receptor (RyR) (Hoch et al. 1998), the Ca^{2+}-ATPase of the sarcoplasmic reticulum (SERCA2a) and phospholamban (Hoch et al. 1998).

Interestingly, the subcellular distribution of SERCA2a and phospholamban corresponds to that in cardiomyocytes (Hoch et al.1998). CaMKIIδ isoforms are also expressed in H9c2 cardiomyoblasts (Hoch et al. 1998). Moreover, Western blot analysis revealed the presence of PKCα, βI, ε, δ, and ζ (Chen and Chen 1999). Therefore, it was concluded that H9c2 cardiomyoblasts may serve as a useful model for transmembrane signaling pathways of cardiomyocytes (Hescheler et al. 1991).

The presence of a V_{1a} receptor/G_q-protein/phospholipase Cβ (PLCβ) pathway in H9c2 cardiomyoblasts has already been demonstrated (Chen and Chen 1999). Treatment of the cells with vasopressin led to a dose-dependent stimulation of inositol phosphate formation as well as to the activation of phospholipase A2 and PKCα, βI, ε, and δ. A subsequent activation of the p42 MAPK was also observed (Chen and Chen 1999). Moreover, fluorometer tracings of intracellular Ca^{2+} in H9c2 cardiomyoblasts in response to vasopressin demonstrated a biphasic change of Ca^{2+} levels: a peak (phasic response) followed by a lower Ca^{2+} plateau (tonic response). It was shown that the extracellular Ca^{2+} is responsible for the tonic phase whereas intracellular Ca^{2+} sources define the phasic peak (Chen and Chen 1999).

Furthermore, H9c2 cardiomyoblasts have been suggested to be a convenient model for the analysis of vasopressin-induced myocyte hypertrophy. It was shown that vasopressin treatment (1μM) of H9c2 cardiomyoblasts led to an approximate 1.5-fold stimulation of protein synthesis (Brostrom et al. 2000). In addition, the number of cells did not differ significantly between untreated and vasopressin-treated preparations showing that hypertrophy had taken place. Moreover, incubation with vasopressin did not promote entry into the cell cycle, since 84% of the cells were found to be in G_o after 24 hours of serum starvation, regardless of exposure to vasopressin (Brostrom et al. 2000).

Leucine pulse incorporation experiments revealed that hypertrophy occured mainly within the first 24 hours and was completed at 48 hours (Brostrom et al. 2000). It was demonstrated that the stimulation of protein synthesis was independent of changes in extracellular Ca^{2+} but was dependent on gene transcription, since treatment with actinomycin D led to the elimination of the increase in protein (Brostrom et al. 2000).

Activation of the PKC appeared to be essential for vasopressin-induced hypertrophy because a PKC pseudosubstrate sequence inhibited translational upregulation (Brostrom et al. 2000).

1.1.3 Hypertrophic signaling pathways

1.1.3.1 Calcium is a key factor in cardiac hypertrophy

Calcium is a major second messenger in cardiac muscle (Zhang and Brown 2004) and is a key mediator of the hypertrophic adaptational mechanisms (Passier et al. 2000) in response to changes in wall stress as well as a result of neurohumoral and growth factors. The rise of intracellular calcium in cardiomyocytes is caused by calcium entry from extracellular stores through voltage-dependent L-type Ca^{2+} channels and the subsequently Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum through the ryanodine channels (Tsien and Wheeler 1999). In primary cardiomyocytes, a hypertrophic response can be induced by elevation of extracellular Ca^{2+} (Lapointe et al. 1990), stimulation with Ca^{2+} agonists (Sei et al. 1991) or treatment with Ca^{2+} ionophores (Sonnenberg 1986). Also, disturbances in Ca^{2+} handling have been associated with altered contractility of cardiomyocytes caused by aberrant expression of sarcomeric proteins (Frey et al. 2000). On the other hand, GPCRs classically increase Ca^{2+} levels by producing IP3, which induces Ca^{2+} release from the endoplasmic reticulum (ER) via the IP3 receptor (Berridge 1993). Increased levels of intracellular Ca^{2+} can transduce signals through a number of Ca^{2+} regulated enzymes such as calcineurin and Ca^{2+}-calmodulin-dependent protein kinases (Fig. 1) (Marks 2003).

1.1.3.2 Calcium-calmodulin-dependent signaling pathways

Calmodulin is an important protein acting as a signal transducer in response to a rise in intracellular calcium (Chin and Means 2000). The binding of calcium ions leads to a conformational change and subsequent activation of calmodulin (Klee 1988). Calmodulin is then able to bind target proteins leading to their activation or inhibition. It has been shown that overexpression of calmodulin in a transgenic mouse model leads to severe hypertrophy of the heart (Gruver et al. 1993). Also, the calmodulin inhibitor W-

7 was able to prevent hypertrophy of cultured cardiomyocytes (McDonough and Glembotski 1992).

1.1.3.2.1 Calcineurin-NFAT signaling

Calcineurin is a serine-threonine phosphatase and can be found in many tissues (Olson and Williams 2000) including the heart (Molkentin 2000). It is a heterotrimer and consists of a catalytic A subunit, a regulatory B subunit and calmodulin (Klee et al. 1988). The calcium-calmodulin complex can activate calcineurin by binding to the catalytic A subunit, leading to a conformational change and subsequent displacement of an autoinhibitory domain from the active site of the enzyme (Aramburo et al. 2004; Klee et al. 1998). A variety of endogenous factors can also influence calcineurin activity. The C-terminal domain of Cabin 1/Cain can regulate calcineurin as a non-competitive inhibitor (Lai et al. 1998). AKAP79 (A-Kinase Anchoring Protein-79) binds calcineurin leading to its inactivation (Coghlan et al. 1995). Recently, the myocyte-enriched calcineurin-interacting proteins (MCIP) have been identified as a new family of calcineurin inhibitory proteins, which are thought to regulate calcineurin activity in the heart (Fuentes et al. 1995; Rothermel et al. 2000; Kingsbury and Cunningham 2000). Interestingly, MCIP expression is regulated by calcineurine itself (Yang et al. 2000). Pharmacological inhibitors of calcineurin include cyclosporin A (CspA) and FK506. By forming a complex with cyclophilin or FKBP12, respectively, and subsequently binding to the catalytic subunit, both of them inhibit calcineurin (Leinwand 2001; Shaw et al. 1995).

The ubiquitously expressed nuclear factor of activated T cells (NFAT) proteins are main targets of calcineurin (Lyakh et al. 1997). NFAT proteins regulate cytokine expression in T cells (Rao et al. 1997) and are involved in cardiac valve development (NFAT2) and in heart hypertrophy (NFAT3) (Ranger et al. 1998; Hoey et al. 1995). NFAT proteins possess a regulatory domain, consisting of a targeting motif for calcineurin and a nuclear localization signal, and a central Rel-like domain, which dictates binding specificity (Aramburu et al. 1998; Chen et al. 1998). NFAT is located in the cytoplasm in a hyperphosphorylated state. Calcineurin binds to and dephosphorylates NFAT, thereby revealing the nuclear localization signal and leading to its translocation into the nucleus (Okamura et al. 2000). Here, NFAT can bind to the respective consensus binding site of

a target gene and either activate or repress its expression. Since NFAT-DNA interaction is generally of a weak nature, interaction of NFAT with other nuclear transcription factors such as AP-1, GATA-4 and MEF2 also takes place (Hogan et al. 2003).

An active calcineurin-NFAT pathway in cardiomyocytes has been demonstrated (Molkentin et al. 1998). Moreover, transgenic mice expressing activated forms of calcineurin or NFAT3 in the heart showed enormous cardiac enlargement and subsequently developed heart failure (Molkentin et al. 1998). Also, in the heart of patients with hypertrophic cardiomyopathy, calcineurin activity was increased (Ritter et al. 2002). It was demonstrated that the pharmacological calcineurin inhibitor CspA prevented hypertrophy of neonatal rat cardiomyocytes that had been stimulated with Ang II or phenylephrine (PE) (Olson and Williams 2000). Moreover, several studies showed that the inhibitors CspA and FK506 lead to an inhibition or attenuation of cardiac hypertrophy in rodents (Olson and Williams 2000; Molkentin 2000; Leinwand 2001). However, other *in vivo* experiments did not lead to the same effects or even worsened the phenotype (Fatkin et al. 2000). In transgenic mice overexpressing a constitutively active calcineurin and the endogenous calcineurin-binding protein MCIP, cardiac hypertrophy, reexpression of fetal genes and progression to dilated cardiomyopathy was inhibited (Rothermel et al. 2001).

1.1.3.2.2 Multifunctional CaMK signaling

Calcium-calmodulin-dependent protein kinases (CaMKs) are a family of serine/threonine kinases. Myosin light chain kinase, phosphorylase kinase and elongation factor-2 kinase (originally termed Ca^{2+}-calmodulin-dependent protein kinase (CaMK) III) are CaMKs phosphorylating a single substrate (Hudmon and Schulman 2002; Nairn et al. 1985). CaMKI, CaMKII and CaMKIV are multifunctional CaMKs and phosphorylate several substrates (Lee and Edelman 1994; Tokumitsu et al. 1995). KN93 is a membrane permeant inhibitor of CaMKI, II and IV (Hook and Means 2001). KN92 is a congener of KN93 but without CaMK inhibitory activity and is used as an experimental control (Tombes et al. 1995). It has been suggested that an important function of CaMKs is to sense alterations of Ca^{2+} signals and to mediate changes in Ca^{2+} regulatory proteins and transcriptional processes (Zhang and Brown 2004). CaMKI is expressed in a variety of tissues and can be found in the cytoplasm in mammalian

cells (Soderling 1999). Even though CaMKI is also expressed in the heart, it is not upregulated during hypertrophy (Uemura et al. 1998; Colomer et al. 2003). CaMKIV is expressed in neuronal tissues, T lymphocytes and testis (Soderling 1999), and in cardiac tissue at low levels (Braun and Schulman 1995; Heist and Schulmann 1998). CaMKIV can be found in the cytoplasm, but seems to be mainly localized in the nucleus (Jensen et al. 1991). CaMKI and IV are monomers (Nairn and Greengard 1987; Cruzalegui and Means 1993). At least three isoforms of CaMKI and two isoforms of CaMKIV can be found (Yokokura et al. 1997; Means et al. 1991; Sakagami and Kondo 1993). They are activated through phosphorylation by upstream CaMK kinases (CaMKKs) following their binding to Ca^{2+}-calmodulin (Lee and Edelman 1994; Tokumitsu et al. 1995). The CaMKKs themselves are calcium-calmodulin-dependent enzymes. In addition, CaMKIV can undergo autophosphorylation (Chatila et al. 1996). CaMKII is a ubiquitously expressed homo- or heteromultimer consisting of 8-12 subunits, which are encoded by the genes α, β, γ and δ (Braun and Schulman 1995; Kanasaki et al. 1991). Both the δ and γ subunit are present in the heart (Tobimatsu and Fujisawa 1989). However, the δ subunit seems to be the predominant isoform in the animal and human heart (Edman and Schulman 1994; Mayer et al. 1995; Hoch et al. 1999). Alternative splicing leads to variations in CaMKII subunits, some of which contain nuclear localization signals (NLS). CaMKII, mainly consisting of δB subunits, leads to its nuclear localization, whereas δC subunits localize CaMKII to the cytoplasm (Srinivasan et al. 1994; Edman and Schulman 1994; Ramirez et al. 1997). However, CaMKI and IV can inhibit nuclear localization of CaMKII by phosphorylating a specific residue C-terminal to the NLS (Heist et al. 1998). Binding to Ca^{2+}-calmodulin results in activation of CaMKII by relief of autoinhibition and subsequent autophosphorylation (Braun and Schulman 1995). Through the autophosphorylation, CaMKII can give a prolonged response to transient Ca^{2+} signals and is able to sense oscillations of cellular Ca^{2+} (Soderling et al. 2001).

Recent studies have indicated that CaMKs are involved in cardiac hypertrophy. Stimulation of rat cardiomyocytes with PE resulted in CaMK-dependent expression of the hypertrophic marker atrial natriuretic factor (ANF), since a CaMK inhibitor led to inhibition of this effect (Sei et al. 1991). Endothelin-1 (ET-1) increased the activity of CaMKII in cardiomyocytes (Zhu et al. 2000). Overexpression of the wild type δB and not

the δC CaMKII isoform in neonatal rat ventricular myocytes led to increased ANF expression and an enhanced transcriptional response to PE (Ramirez et al. 1997). Moreover, transient overexpression of constitutively active CaMKI and CaMKIV in neonatal cardiomyocytes resulted in a hypertrophic response (Passier et al. 2000). Ca^{2+}-dependent signaling has been implicated in hypertrophic myocyte growth suggesting a direct link between cardiac growth and the function of Ca^{2+} handling proteins (Sadoshima and Izumo 1995; Olson and Molkentin 1999). CaMKII regulates numerous intracellular Ca^{2+} handling proteins such as phospholamban and SERCA2 (Simmermann et al. 1986; Xu et al. 1993). A number of animal models also proved the involvement of CaMKs. Transgenic overexpression of CaMKIV in mice resulted in cardiac hypertrophy (Passier et al. 2000). Increased CaMKIIδ expression (Hagemann et al. 2001) and CaMKII activity (Boknik et al. 2001) was demonstrated in the hypertrophied myocardium from spontaneously hypertensive rats. It has been shown that pressure-overload hypertrohy induced by transverse aortic constriction in mouse hearts or by acute increases in pressure in perfused rat hearts is concomitant with an increase in CaMKII expression and activity (Colomer et al. 2003; Zhang et al. 2003; Saito et al. 2003). Also, pressure-overload appeared to result in differential regulation of the cytoplasmic δC and the nuclear δB CaMKII isoforms (Colomer et al. 2003; Zhang et al. 2003). Analysis of human cardiac tissue has shown that CaMKII activity (Kirchhefer et al. 1999) and CaMKIIδ expression (Hoch et al. 1999) are increased in patients with dilated cardiomyopathy. Moreover, it has been demonstrated that CaMKII is involved in the regulation of a number of hypertrophic marker genes such as ANF (Ramirez et al. 1997) and β-myosin heavy chain (MHC) (Zhu et al. 2000). In addition, CaMKs play a role in the regulation of transcription factors such as cAMP-response element-binding protein (CREB) (Sheng et al. 1991), CAAT-enhancer binding protein (C/EBP) (Wegner et al. 1992) and activating transcription factor-1 (ATF-1) (Sun et al. 1996). Interestingly, CaMKII and IV phosphorylation of CREB can result in opposite transcriptional effects (Zhang et al. 2003).

1.1.3.2.2.1 Signaling via MEF2

Myocyte enhancer factor-2 (MEF2) is a transcription factor which integrates multiple Ca^{2+}-calmodulin-dependent signaling pathways in muscle cells (McKinsey et al. 2002).

Four genes (MEF2A-D) code for the different MEF2 proteins but only MEF2A and MEF2D are expressed significantly in the adult heart. However, the cardiac specific MEF2 proteins show only basal levels of transcriptional activity (Naya et al. 1999). Upon the binding of cofactors, their activity is either stimulated (MEF2, MyoD, GATA, NFAT, ERK5) or repressed (HDAC4, 5, 7 and 9, cabin) (Im and Rao 2004). MEF2 activity is controlled by direct association with histone deacetylases (HDACs). HDACs bind to transcription factors such as MEF2 and subsequently deacetylate nucleosomal histones, leading to chromatin condensation and transcriptional repression. Conversely, histone acetyltransferases relax chromatin and thereby activate target genes (McKinsey et al. 2002). Three classes of HDACs exist and high levels of class II HDACs are found in striated muscle. N-terminal extensions in these HDACs interact with specific cofactors and transcription factors. Phosphorylation of specific sites within this part of the histone deacetylase (HDAC) regulates these associations (McKinsey et al. 2002). This phosphorylation can be carried out by CaMKs and results in HDAC export to the cytoplasm. Hence MEF2 is released and is able to associate with coactivators and stimulate transcription of MEF2-dependent genes (McKinsey et al. 2000).

MEF2 factors have been connected with developmental growth and postnatal hypertrophy of the heart (Lin et al. 1997; Kolodziejczyk et al. 1999). It has been shown that pressure-overload and calcineurin activation result in activation of a kinase that phosphorylates the class II HDACs (Zhang et al. 2002). CaMKIV mediates a hypertrophic response upon HDAC phosphorylation, leading to nuclear export of the HDAC and release of active MEF2 from the HDAC/MEF2 complex (Passier et al. 2000). CaMK IV has also been reported to directly phosphorylate MEF2, but it has not been determined whether this is responsible for transcriptional activation of the protein (Blaeser et al. 2000). Also, in PE-induced hypertrophy of primary neonatal cardiomyocytes CaMKIV leads to dissociation of HDACs from MEF2 resulting in its activation (Lu et al. 2000). Furthermore, adenoviral-mediated expression of mutant HDAC5 or HDAC9 lacking the regulatory phosphorylation sites led to resistance of cardiomyocytes in response to serum- or PE-induced cardiomyocyte hypertrophy (Frey and Olson 2003). Transgenic mice lacking HDAC9 demonstrated normal cardiac size and function at early age but developed spontaneous cardiac hypertrophy later (Frey and Olson 2003). In addition, germline deletion of a class II HDAC results in an increase in MEF2 activity and in extreme hypertrophy in the adult hearts (Zhang et al. 2002).

1.1.3.3 Signaling via PKC

The protein kinase C (PKC) family of calcium- and/or lipid-activated serine/threonine kinases represents a downstream signal of almost all membrane-associated signal transduction cascades (Molkentin and Dorn II 2001) and consists of about twelve different isoenzymes (Hayashi et al. 1999). The PKC is composed of a regulatory domain, which interacts with calcium, DAG and phosphatidylserine and a catalytic domain interacting with ATP and other proteins (Toullec et al. 1991). Activation of the membrane-bound PKC leads to its translocation to specific subcellular sites through interaction with docking proteins (Mochly-Rosen et al. 1991). Transcription factors can be target proteins of PKC phosphorylation (Clerk et al. 1994). The cell permeable inhibitor bisindolylmaleimide (BIM) binds competitively at the ATP binding site (Toullec et al. 1991) and inhibits membrane and cytosolic PKC (Budworth and Gescher 1995). Furthermore, BIM is highly selective for PKC (Toullec et al. 1991).

Different PKC isoforms have been implicated in cardiac hypertrophy (Frey and Olson 2003). Overexpression of PKCβ in mouse hearts leads to cardiac hypertrophy and sudden death (Bowman et al. 1997). In failing human hearts, activity of this isoform was found to be increased (Bowling et al. 1999). However, other studies could not confirm these results (Huang et al. 2001; Tian et al. 1999). Moreover, PKCβ knock out mice showed an unchanged hypertrophic response due to pressure-overload or phenylephrine, suggesting that PKCβ is not necessary for the development of cardiac hypertrophy (Roman et al. 2001). It was demonstrated that a constitutively overexpressed PKCε led to concentric cardiac hypertrophy in mice (Takeishi et al. 2000). Another study demonstrated that a transgenic mouse model with a modest rise in PKCε led to a cardioprotective phenotype, whereas a second transgenic mouse with a more drastic increase in PKCε activity resulted in cardiac hypertrophy and subsequent heart failure (Pass et al. 2001). Furthermore, only PKCα has been shown to be necessary for cardiomyocyte hypertrophy *in vitro*, partly mediated by an extracellularly responsive kinase (ERK)-dependent signaling cascade (Braz et al. 2002). Transgenic overexpression of a PKC-dependent MAPK kinase that activates ERK1/2 evoked a hypertrophic phenotype of the heart (Bueno et al. 2000).

1.1.3.4 Signaling via MAPK

Mitogen-activated protein kinases (MAPKs) are initiated by cell surface receptors such as gp130 through a protein kinase cascade (Davis 1993, Frey and Olson 2003). This cascade involves the MAPK kinase kinases (MAPKKK: c-Raf-1, c-Mos and Ste II), which phosphorylate MAPK kinase (MAPKK) isoforms, which in turn leads to phosphorylation and activation of MAPKs (Davis 1993). The MAPKs consist of three major subfamilies: extracellularly responsive kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 MAPKs (Frey and Olson 2003). The JNKs and p38 MAPKs are stress-responsive MAPKs because in addition to activation by anabolic stimuli and agonists of G-protein-coupled receptors, they are also stimulated by pathological stress such as ischemia or cytotoxic agents (Sugden and Clerk 1998). The MAPKs regulate a number of transcription factors and thereby transduce an external stimulus (i.e. mitogen stimulation) to the nucleus (Frey and Olson 2003). In fact, p38 phosphorylates MEF2 (Han et al. 1997) and NFAT3 (Yang et al. 2002), two transcription factors that are involved in the regulation of gene expression under hypertrophic conditions. Also, p38 activity is induced by pressure-overload (Takeishi et al. 2001) and ET-1/PE stimulation (Clerk et al. 1998; Ueyama et al. 1999). Mechanical stretching (Komuro et al. 1996) or stimulation by ET-1 (Choukroun et al. 1998), PE (Ramirez et al. 1997) or Ang II (Yano et al. 1998) leads to phosphorylation of c-Jun N-terminal kinase (JNK) in cardiomyocytes. The role of ERK1/2 in hypertrophy is not clear. Blockage of ERK1/2 signaling did not result in ANF inhibition in neonatal cardiomyocytes (Post et al. 1996). Also, transgenic overexpression of G_q did not lead to ERK1/2 activation (D'Angelo et al. 1997). On the other hand, it has been shown that ERK1/2 depletion led to an attenuated hypertrophic response in cardiomyocytes (Glennon et al. 1996). Moreover, overexpression of MEK1, a MAPKK that only activates ERK1/2 (and not JNKs and p38 MAPKs) leads to cardiac hypertrophy (Bueno et al. 2000).

1.1.3.5 Cross-talk

A number of reports indicate that multiple signaling networks play a role in the development of hypertrophy (Molkentin and Dorn II 2001). One important aspect in the integration of cardiac signaling is the cross-talk between parallel signaling cascades (Bueno et al. 2002). For example, the existence of a conserved and interconnected

regulatory circuit between calcineurin, PKC and JNK MAPK has been suggested. Transgenic overexpression of calcineurin led to activation of JNK MAPK and several PKC isoforms. Likewise, cardiomyocyte hypertrophy induced by adenovirally mediated expression of calcineurin was prevented by JNK or PKC inhibition (De Windt et al. 2000a; De Windt et al. 2000b). In addition, treatment of pressure overloaded rat hearts with the calcineurin inhibitor CspA inhibited PKCα and JNK activation (De Windt et al. 2000a). Concerning the calcineurin downstream effector NFAT, it has been shown that its rephosphorylation is regulated by a number of kinases, e.g., gylcogen synthase kinase 3β, JNK MAPK, p38 MAPK and protein kinase A (Aramburu et al. 2004), leading to its nuclear export and transcriptional inactivity. Conversely, activated ras has been demonstrated to promote nuclear localization of NFAT3. Likewise, a dominant-negative ras-mutant was able to diminish the PE-induced increase in NFAT activity (Ichida and Finkel 2001). Moreover, alternative transcription factors may be reponsible for the nuclear events downstream of calcineurin. It has been demonstrated that calcineurin activation leads to upregulation of a MEF2-activated reporter transgene in the hearts of transgenic mice (Passier et al. 2000).

1.1.4 Gene expression during cardiac hypertrophy

Induction of immediate-early genes such as proto-oncogenes (i.e c-fos, c-jun and c-myc) and heat shock protein genes (e.g. hsp 70*)* (Mulvagh et al. 1987; Komuro et al. 1988; Izumo et al. 1988) and, later, the reinduction of the fetal gene program constitute quantitative changes of gene expression during the hypertrophic process. The reexpression of fetal genes in rodents involves the transition of cardiac α-actin to skeletal α-actin (Schwartz et al. 1986), reexpression of ANP (Izumo et al. 1988; Mercadier et al. 1989) and a shift of the expression from α-MHC to β-MHC (Schwartz et al. 1986). Similarly, in the human ventricle, cardiac hypertrophy is associated with reexpression of the atrial-specific essential myosin light chain (ALC-1) (Morano et al. 1997).

Shifts in isogene expression concerning proteins involved in energy metabolism such as lactate dehydrogenase and creatine phosphokinase have been found (Revis et al. 1977; Meerson and Javich 1982). Furthermore, fatty acid oxidation, which the adult heart

usually employs for ATP production, is suppressed and an increase in glucose utilization takes place (Depre et al. 1998), leading to a decrease in oxygen consumption per generated ATP (Barger and Kelly 2000).

Downregulation of genes encoding membrane proteins such as the sarcoplasmic reticulum Ca^{2+}-ATPase also takes place (Anger et al. 1998; Nagai et al. 1989).

Moreover, the increase in myocardial mass as the key process of cardiac hypertrophy is a direct consequence of an enhanced protein synthesis, in particular of sarcomeric proteins (Swynghedauw 1999; Sadoshima and Izumo 1997).

1.1.5 Type II myosin

Muscle contraction is triggered by the cyclic interaction of the molecular protein type II myosin with the actin filament to produce tension or shortening. Multiple myosin isoforms exist in all eukaryotic cells and form a structurally and functionally diverse superfamily, consisting of a minimum of 18 distinct families (Berg et al. 2001). Type II myosin is a hexamer, consisting of two heavy chains (MHC) each associated with two light chains (MLC, 18-28 kD) (Fig. 2). The MLC types are classified into essential MLC (MLC-1) and regulatory (phosphorylable) MLC (MLC-2) (Weeds and Pope 1971). Together with calmodulin and troponin C, both types belong to the superfamily of EF-hand Ca^{2+} binding proteins (Moncrief et al. 1990). There are two MHC (α- and β-MHC) (Leinwand et al. 1983) and five MLC isoforms in the human heart. Of these MLC, three are regulatory (two ventricular-specific (VLC-2 and VLC-2*), one atrial-specific (ALC-2)) (Price et al. 1980) and two are essential (one ventricular-specific (VLC-1), one atrial-specific (ALC-1)) (Kurabayashi et al. 1988). In the normal adult human ventricle of the heart, the β-MHC, VLC-2, VLC-2* and VLC-1 are expressed (Morano 1999). Within the MHC, three structural and functional domains can be distinguished: 1) the N-terminal, most conserved globular motor domain where actin-and ATP-binding sites are located 2) the regulatory domain also called the neck, containing the motifs that define the light chain-binding region (non-covalent binding) 3) the C-terminal domain or α-helical tail, the most diverse part of the heavy chain that is responsible for dimerization and filament formation (Fig. 2) (Sellers 1999).

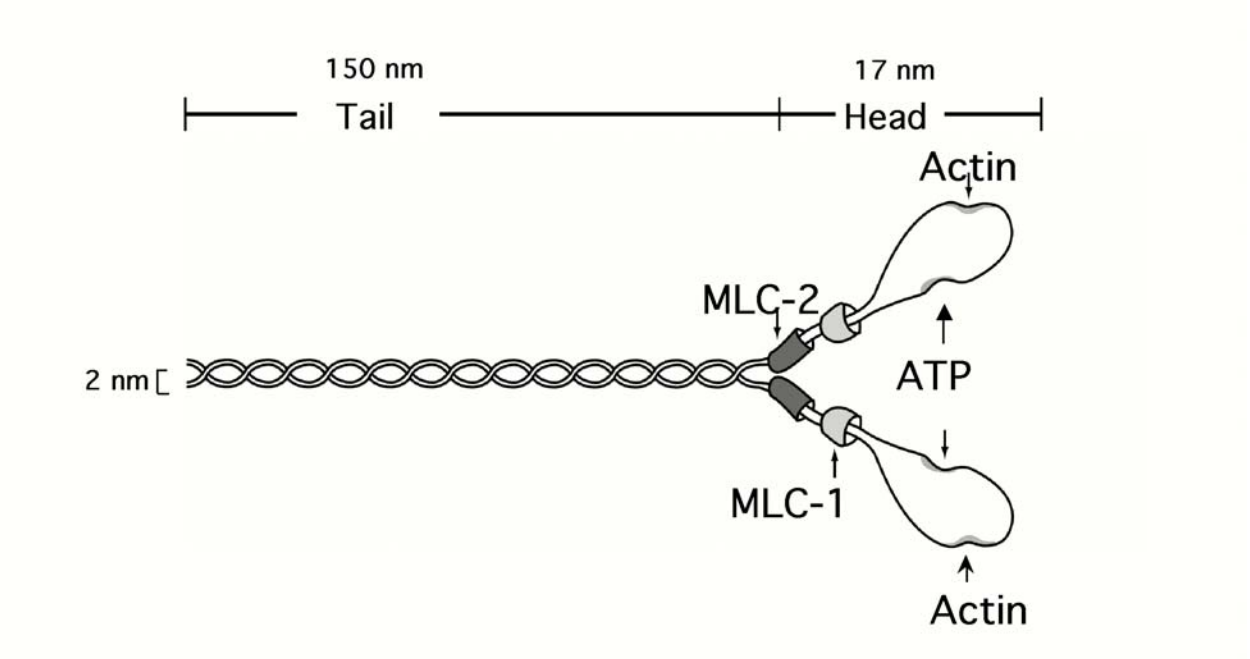

Figure 2. Scheme of a type II myosin molecule
The myosin type II molecule consists of two globular heads, each joined to an α-helical tail. The head region contains the actin- and ATP-binding sites (motor domain) as well as the non-covalently bound MLC-1 and MLC-2 (neck region).

The MHC is loaded with either ATP or ADP and inorganic phosphate (P_i) and binds to the N-terminus of actin as a cross-bridge. Myosin can be weakly (with low affinity) or strongly (with high affinity) attached (Eisenberg and Hill 1985; Brenner 1988). During the transition from the weakly to the strongly attached state, force is generated, which is asociated with the release of P_i (Dantzig et al. 1992). Recently, a new model for the interaction of myosin and actin has been proposed. Here, elastic bending of the light chain binding domain, the lever arm, leads to the generation of force as opposed to the tilting of the catalytic domain described in a former model (Eisenberg and Hill 1985; Holmes 1997; Irving et al. 1995; Lombardi et al. 1995).

1.1.5.1 Expression regulation of the human atrial myosin light chain 1 (hALC-1)

In the initiation process of mRNA synthesis, RNA polymerase II forms a preinitiation complex at the promoter of a gene together with an ordered assembly of general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH). These factors assist

the polymerase in locating promoters through interactions with factors bound to the promoters and/or enhancer sequences. A promoter comprises the 5' flanking region of a gene and can be roughly defined as the sum of DNA sequences necessary for transcription initiation (Pollard and Earnshaw 2004). However, a distinction is made between basal levels of transcription (regulatory factors are not present) and regulated (activated or repressed) transcription, which is directed by specific gene regulatory proteins. The TATA box is a conserved consensus sequence located approximately 30 bp upstream of the transcription start site and belongs to the basal promoter machinery. Additionally, the initiator, a less conserved promoter element, is found close to the transcription start site of many genes (Pollard and Earnshaw 2004). Enhancers augment the rate of initiation from a basal promoter 1) even if they are located very far from the basal promoter, 2) whether their location is internal to or downstream of the promoter and 3) in either orientation relative to the promoter. Furthermore, a number of specific transcription factors can bind to particular DNA sequences, thereby turning genes on or off (Pollard and Earnshaw 2004). A conformational change in the polymerase-promoter complex leads to an open complex with unpaired DNA. Hereby, RNA polymerase gains access to nucleotide bases that are complementary to the start of the message. A phosphodiester bond is formed between the first two complementary ribonucleotides, after which the polymerase translocates one base and repeats the process of phosphodiester bond formation, leading to elongation of the nascent RNA (Pollard and Earnshaw 2004). During transcription processing of the primary nuclear transcript takes place, such as methylation of the 5' end of the mRNA (capping). This 5' cap is stable throughout the life of an mRNA and protects the 5' end against attack from nucleases. Furthermore, a polyadenylation signal leads to the cleavage of the mRNA chain, releasing it from the transcription complex, and subsequently to the addition of 50 to 200 adenine residues (poly-A tail) to the 3' end. This is suggested to protect the mRNA from degradation in the cytoplasm. Most messages are also spliced to remove introns, which are non-coding sequences. Untranslated regions (UTRs) are stretches of RNA upstream of the start codon and downstream of the stop codon that are not translated (Pollard and Earnshaw 2004).

The human ALC-1 gene is located on the long arm of chromosome 17 (Seharaseyon et al. 1990) and is divided into eight exons (Rotter et al. 1991). Whereas exons 1 through 6 are protein-coding exons, exons 7 and 8 represent 3‘ untranslated region (UTR)

(Rotter et al. 1991). In cultured human skeletal muscle cells, different hALC-1 mRNAs with identical protein coding regions but alternative 5‘ and 3‘ UTRs have been found. This can be explained by the fact that ALC-1 transcription can be driven by two different promoters and that splicing can produce two alternative 3‘ UTRs (3’ UTR/7 and 3’ UTR/8) (Fig. 3) (Zimmermann et al. 1990; Rotter et al. 1991). Whereas a putative TATA box is located upstream of the first transcription start site, an A-rich region precedes the distal transcription start site. This sequence stretch might serve as a TATA box though. In fetal and adult human cardiac tissue, both transcription start sites are used and the corresponding mRNAs can contain either the proximal or the distal 3‘ UTR (Rotter et al. 1991).

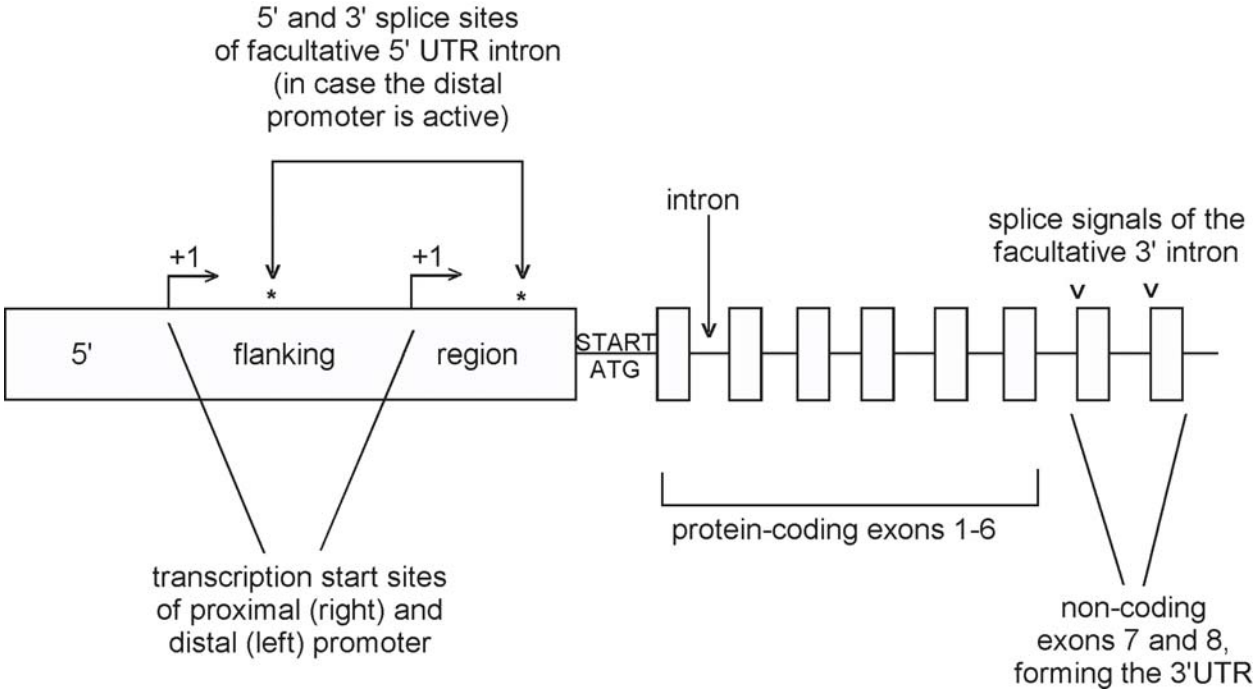

Figure 3. Scheme of the structure of the hALC-1 gene (c.f. Rotter et al. 1991). The 5’ flanking region of the hALC-1 gene contains two transcription start sites. Thus, the hALC-1 gene can be transcribed from two different promoters (proximal and distal). In case transcription is driven by the distal promoter, a facultative 5’ intron can be spliced out. Exons 1 through 6 are protein-coding exons. Exons 7 and 8, both of which contain a putative polyadenylation signal, are not translated and represent the 3’ untranslated region. Within this region a facultative 3’ intron can be removed. +1, transcription start site; UTR, untranslated region; *, splice sites of facultative 5’ intron; ∨, splice signals of the facultative 3’ intron; START ATG, translation start site.

Sequence analysis of 2 kb of 5‘ flanking region revealed a variety of consensus binding sites for transcription factors within the hALC-1 promoter (Rotter et al. 1991). Two sequence motifs, which show homology to glucocorticoid responsive elements, were found. Also, a sequence which is homologous to the MEF1-binding enhancer element was identified (Rotter et al. 1991). This binding site was originally found in the muscle creatine kinase gene (Buskin and Hauschka 1989) and has been reported to bind the myogenic determination factor MyoD1 (Lassar et al.1989). An „MLC sequence“ motif, which has been described to be common for the fast, ventricular and atrial MLC genes of mouse (Cohen et al. 1988), was also found in the hALC-1 promoter sequence (Rotter et al. 1991). This sequence conservation indicates an important role of the motif in the transcription of MLC genes, but until now this role has not been defined (Rotter et al. 1991). In addition, a putative transcription factor binding site and enhancer element, which have been found in the promoter region of the mouse MLC1/MLC3 gene (Daubas et al. 1985), were identified in the hALC-1 promoter (Rotter et al. 1991). The enhancer element shares 100% homology with the core sequence of the major enhancer of polyoma virus and of the adenovirus E1a gene (Daubas et al. 1985). The function of these sequence motifs is still unresolved (Rotter et al. 1991). Moreover, a binding site for the zinc-finger trancription factor GATA-4 was identified within the hALC-1 promoter (Ritter et al. 1999b). GATA has been reported to be involved in the development of heart hypertrophy (Herzig et al. 1997). It can also interact with a member of the NFAT family, NFAT3, thereby increasing its DNA binding activity (Molkentin et al. 1998). However, there is also a NFAT binding sequence motif in the hALC-1 promoter, suggesting that NFAT is capable of directly activating ALC-1 transcription (Ritter et al. 1999b). Furthermore, an E-box exists in the promoter region of the hALC-1 gene (Ritter et al. 1999b). Basic helix-loop-helix (bHLH) proteins can dimerize with E12 transcription factors and recognize the E-box consensus sequence of target genes (Hollenberg et al. 1995). These bHLH transcription factors play a role in the differentiation and maturation of skeletal muscle (Olson and Klein 1994). Two members of this family are the so-called HAND proteins eHAND (also HAND1, Thing1 or HXT) and dHAND (also HAND2, Thing2 or HED) (Srivastava et al. 1995). They have been described to influence cardiac looping and ventricular specification and growth (Thomas et al. 1998).

In the adult human ventricle, the amount of ALC-1 mRNA is below a threshold level which is necessary for translation. However, in the hypertrophied ventricle of patients with tetralogy of Fallot (TOF) and hypertrophic obstructive cardiomyopathy (HOCM) ALC-1 mRNA is upregulated (Ritter et al. 1999b). Also, in these patients endogenous antisense ALC-1 mRNA was detected next to sense ALC-1 mRNA. It was shown that the ALC-1 translation product decreased with increasing amounts of ALC-1 antisense mRNA. Specifically, in comparison with HOCM, TOF patients showed a higher level of ALC-1 mRNA, which was proposed to be an adaptational mechanism in order to overcome the high amount of antisense ALC-1 mRNA and to maintain the sense ALC-1 mRNA at a treshold level allowing its translation (Ritter et al. 1999b). Interestingly, a positive correlation between HAND mRNA and ALC-1 mRNA was found in the hypertrophied ventricles of these patients (Ritter et al. 1999b). Since it has been shown that E-boxes are sufficient for ALC-1 transcription in mouse (Catala et al. 1995), it was suggested that the hALC-1 gene might be a downstream target of HAND transcription factors (Ritter et al. 1999b).

During the biochemical process of translation, sequences of nucleotides in an mRNA are translated into the sequence of amino acids in a polypeptide chain. The protein-coding region is comprised of codons, which are successive triplets of three nucleotides. These specify the sequence of amino acids (Pollard and Earnshaw 2004). During the initiation phase, the small ribosomal subunit and an initiator tRNA bind the initiation codon (AUG) of an mRNA, which begins all polypeptide chains. Eukaryotic initiation factors (eIFs) are proteins which participate in the initiation step. Throughout the elongation phase, the tRNAs carry the amino acids to the ribosome where they are matched with the corresponding mRNA codons and incorporated into the growing polypeptide (Pollard and Earnshaw 2004). During translation of an mRNA, the small and the large ribosomal subunit bind together. In the cavity between these subunits, mRNA decoding and synthesis of the polypeptide are carried out. Peptide synthesis is stopped by any of the three termination codons (UAA, UGA, UAG) resulting in the release of the polypeptide from the ribosome. A number of soluble proteins enhance the rate or fidelity of protein synthesis (Pollard and Earnshaw 2004).

Human ALC-1 protein expression is developmentally regulated. Large amounts of ALC-1 protein are found in skeletal muscle and in the whole heart of human embryos (Barton

and Buckingham 1985). This expression decreases to undetectable levels during early postnatal development in the ventricle, though it persists in the atrium for the entire life (Fallot et al. 1888). The hALC-1 is reexpressed in the hypertrophied right ventricle of patients with the congenital heart disease TOF (Auckland et al. 1986). ALC-1 protein is also expressed in the hypertrophied left ventricles of patients with ischemic, dilative (DCM) and hypertrophic (HCM) cardiomyopathy (Schaub et al. 1984; Ritter et al. 1999b; Morano et al. 1997). Surgical intervention leading to a normalization of hemodynamics decreased ALC-1 expression in the hypertrophied left ventricles (Sütsch et al.1992). The amount of ALC-1 protein in the diseased ventricles is heterogenous and varies between 0-35% of total MLC-1. However, patients with HOCM seem to express a higher amount of ALC-1 in their hypertrophied ventricles (Ritter et al. 1999b) than patients with DCM (Morano et al. 1997). Also, in patients with end-stage congestive heart failure (CHF) ALC-1 protein was either undetectable or found only in small amounts (Morano et al. 1996).

1.1.5.2 Functions of hALC-1

Several studies have unravelled the functional impact of ALC-1 reexpression in the heart. Material of the right ventricular infundibulum of patients with TOF was analyzed with regard to the cross-bridge cycling kinetics of the intact sarcomere. It was demonstrated that with increasing amounts of ALC-1 in the skinned fibers 1) the maximal shortening velocity increased significantly and that 2) the half time of tension development decreased (=higher detachment rate and rate of force development) (Morano et al. 1996). This suggests that the expression of ALC-1 in the human heart modulates cross-bridge cycling kinetics and improves the contractile state of the heart. In addition, there was a compensatory downregulation of the VLC-1 expression in order to maintain the normal MLC-1/MLC-2 ratio (Morano et al. 1996). Furthermore, a significant positive correlation between ALC-1 protein expression and the maximal rate of isovolumetric force development was found in the hypertrophied ventricles of patients with HOCM (Ritter et al. 1999a).

The N-terminal peptide 5-14 of ALC-1 has a significantly lower affinity for actin than the corresponding N-terminal VLC-1 peptide (Morano and Haase 1997). Therefore, ALC-1 is a weaker MHC/actin tether in comparison with VLC-1 (Morano et al. 1996). The ALC-

1 N-terminus also contains less charged amino acids than VLC-1 (Fodor et al. 1989). It has been shown that a decrease in charged amino acids also leads to a weaker MHC/actin tether (Sweeney 1995). Moreover, Ca^{2+} has been demostrated to play a role in the conformational status of ALC-1 *in vitro*. It was proposed that during systole of the cardiomyocyte, when a high amount of Ca^{2+} is present, the N-terminus is in an extended conformational state. During diastole, however, only low amounts of Ca^{2+} lead to a bent form of the N-terminus and thereby a dissociation from actin (Stepkowsky 1995; Morano 1999). In addition, it has been suggested that the β-MHC associated with the VLC-1 presents a lever arm with greater stiffness than the β-MHC associated with an ALC-1 molecule. This latter case would again lead to an increased force generation of the sarcomere (Howard and Spudich 1996; Morano 1999).

Transgenic overexpression of the mouse ALC-1 in the ventricle of the mouse heart led to an increase in contractility and relaxation in the absence of a hypertrophic response (Fewell et al. 1998). Moreover, overexpression of the hALC-1 in a transgenic rat model resulted in an increase in developed left ventricular pressure, contraction rate, and relaxation rate. (Abdelaziz et al. 2004). Interestingly, adenoviral gene transfer of hALC-1 to neonatal rat cardiomyocytes did not reveal any differences in oxygen consumption between treated and untreated cells. This is important, because a positive inotropic effect also leads to an enhanced oxygen consumption, which could have deleterious side-effects on the heart, especially in the failing state (Zacharzowsky et al. 2002).

Overall, the studies so far showed that ALC-1 overexpression improved cardiac contractility. Therefore, whereas the rodent displays an isoform shift from α-MHC (high ATPase activity) to β-MHC (low ATPase activity), the human heart reexpresses ALC-1 as a molecular adaptation mechanism to compensate an increased work demand (Ritter et al. 1999a).

1.1.6 Pathophysiological aspects of cardiac hypertrophy

Initially, cardiomyocyte hypertrophy is beneficial for muscular economy (James et al. 2000) leading to normalization of wall stress (Sandler and Dodge 1963). However, epidemiological studies have shown that chronic hypertrophy is associated with a

significant rise in the risk of dilated cardiomyopathy and heart failure, leading to increased cardiovascular mortality (Levy et al. 1990; Casale et al. 1986). In addition, cardiac hypertrophy also occurs in individuals with inherited disease forms, which are linked to mutations in contractile proteins (Marks 2003).

1.1.6.1 Hypertrophic cardiomyopathy caused by mutations

Mutations in genes coding for sarcomeric motor proteins or components of the cytoskeletal architecture and metabolic (mitochondrial) proteins constitute the group of intrinsic hypertrophic stimuli (Maron 1997). Hypertrophic cardiomyopathy (HCM) usually displays autosomal dominant inheritance even though sporadic cases are frequent (Maron 2002). Genetic mutations have been described for α-tropomyosin, cardiac troponin I, essential and regulatory myosin light chains, actin, α-MHC, cardiac troponin T and myosin binding protein C (McKenna et al. 1998), all encoding proteins for the cardiac sarcomere (Maron 2002). Another report also describes a genetic mutation in β-MHC and unconfirmed mutations in titin and Troponin C (Elliott and McKenna 2004). Most mutations are missense mutations that result in a single amino acid substitution within or close to important functional domains (Rayment et al. 1995), leading to reduced contractile function (Redwood et al. 1999). Also, abnormal myocardial bioenergetics have been proposed as the final common pathophysiological pathway (Ashrafian et al. 2003). HCM in infants and young children is associated with congenital malformations and syndromes, inherited metabolic disorders and neuromuscular diseases. Familial disease is less frequent in children than in adults and various modes of inheritance are found (Lipshultz et al. 2003; Nugent et al. 2003). However, disease expression varies not only between unrelated individuals but also within the same family. This indicates that HCM is like a complex inherited trait, which is determined by other genetic and environmental factors (Mogensen et al. 2003; Ackerman et al. 2002).

Hypertrophic cardiomyopathy is a common disease and its main characteristic is „myocardial hypertrophy that is out of proportion to the hemodynamic load“ (Wynne and Braunwald 2001). A major form of HCM includes a disproportionate hypertrophy of the interventricular septum (Maron 1997 and 2002). Even though concentric and apical distributions may arise, the asymmetrical hypertrophy is most common in patients

(Davies and McKenna 1995; Shapiro and McKenna 1983). A predominant feature is the greatly increased myocardial mass and left ventricular wall thickness, which may lead to abnormal stiffness of the heart muscle. Diastolic dysfunction develops with impaired left ventricular relaxation and increased filling pressures (Maron 1997; Nagueh et al. 1999). When the ventricular septum is thickened just below the aortic valve, the blood flow into the aorta may be blocked, which is then termed hypertrophic obstructive cardiomyopathy (HOCM) or left ventricular outflow obstruction. The function of the mitral valve can also be impaired by this thickening, which in turn may lead to distortion of mitral valve movement and a backward blood flow into the left atrium (Nishimura et al. 2003). On the histopathological level, pronounced myocardial hypertrophy can be found together with myocyte disarray. Myocardial fibrosis may also appear (Louie and Edwards III 1994).

Most patients with HCM are asymptomatic or only have few symptoms. The diagnosis is often made incidentally or during family screening. Clinical progression is usually slow. The end result of progressive myocardial hypertrophy is typically cardiac failure and in 10-15% end-stage DCM occurs (Kovacic and Muller 2003). However, the majority of patients with HCM are able to live a normal life (Nishimura et al. 2003). Athletic training can lead to physiological increases in left ventricular mass that theoretically could make diagnosis in young patients confusing (Maron et al. 1995). Ventricle walls, which are as thick as those seen in patients with HCM, are rare. In a small group of people with HCM, arrhythmias can lead to sudden death (Nishimura et al. 2003). Interestingly, HCM is the most common cause of sudden cardiac death in young athletes (Maron et al. 1980).

Medical therapy includes β-blocking agents, which lead to a decreased heart rate with subsequent prolongation of diastolic filling time and a simultaneous decrease in myocardial oxygen demand. Similar effects are seen during treatment with verapamil (Maron 1997; Spirito et al. 1997). The therapy of choice for the prevention of sudden cardiac death is the implantable cardioverter-defibrillator (Maron 2002). Some patients with a significant outflow obstruction will be severely symptomatic. The golden standard is myectomy-myotomy, an operation which involves resection of a small amount of myocardium from the basal septum. Additional mitral valvuplasty or replacement may be considered if mitral regurgitation is problematic (Kovacic and Muller 2003).

1.1.6.2 Dilated cardiomyopathy and heart failure

Dilated cardiomyopathy (DCM) is characterized by an increase in the ventricular chamber size (dilation) and a reduced contractility of the left or both ventricles (Towbin and Bowles 2002; Sangiorgi 2003). In addition, backflow through the mitral valves and abnormal heart rhythms are common. In clinical practice, DCM is heterogenous (Sangiorgi 2003). In progressive cases approximately 50% of the patients die within 5 years of diagnosis, if heart transplantation is not applied (Abelmann and Lorell 1989). Death can be either sudden or caused by pump failure. Therapy includes angiotensin-converting enzyme inhibitors, β-blockers or implantable defibrillators and ventricular assisst devices (Towbin and Bowles 2002).

The interaction of genetic, immunological and acquired (i.e. infective, toxic and metabolic) factors is still considered to lead to the polymorphic clinical picture of idiopathic DCM (Sangiorgi 2003). Genetic disorders may be present in familial but also in non-familial cases (Sangiorgi 2003). They include the X-linked form (X-linked cardiomyopathy and Barth syndrome), the autosomal dominant (pure DCM and DCM associated with cardiac conduction system disease) or recessive form and the mitochondrial form (Grunig et al. 1998; Towbin and Bowles 2002). Affected genes are actin, desmin, δ-sarcoglycan, β-sarcoglycan, cardiac troponin T, β-myosin heavy chain, α-tropomyosin and dystrophin (Towbin and Bowles 2002). Moreover, it is proposed that DCM is caused by enteroviruses, especially those of the Coxsackie B group. However, some of the findings, such as a persistent viral activity in some cases, are still controversial (Martino et al. 1995; Caforio and Goodwin 1993). Also, anti-myocyte autoantibodies are found in the serum of DCM patients, some of which are organ-specific and directed against heavy α- and β-myosin chains, contributing to the hypothesis that DCM can be caused by autoimmunological factors (Neumann 1994; Caforio et al. 1992). Different forms of myocarditis can therefore also be associated with DCM (Sangiorgi 2003). However, the precise mechanisms responsible for the development of DCM and conduction system abnormalities are currently unknown (Towbin and Bowles 2002).

1.2 Aim of the study

Cardiomyocyte hypertrophy is an adaptation of the heart in response to changes in wall stress as well as a result of neurohumoral and genetic factors. The hALC-1 gene is not expressed in the normal ventricle of the adult human heart, but is reexpressed in the overloaded, hypertrophied human ventricle. *In vitro* as well as *in vivo* studies have demonstrated that ALC-1 overexpression improves the contractile state of the heart. Therefore, reexpression of hALC-1 can be considered as a molecular adaptation mechanism to compensate an increased work demand. Detailed information on the regulation of hALC-1 gene expression on the promoter level is lacking. The Ca^{2+}-calmodulin-calcineurin-NFAT signaling pathway has been implicated in the development of heart hypertrophy. It has been demonstrated that the constitutive activation of calcineurin or NFAT leads to massive hypertrophy in transgenic mouse models. Several animal models have also shown that the multifunctional Ca^{2+}-calmodulin-dependent protein kinases CaMKIV and CaMKIIδ are involved in cardiac hypertrophy. Moreover, transgenic overexpression of a PKC-dependent MAPK kinase, which activates ERK1/2, led to cardiomyocyte hypertrophy in the animals. H9c2 cardiomyoblasts are derived from an embryonic rat heart and show characteristics of signaling pathways present in adult cardiomyocytes. It has also been shown that an intact V_{1a}/G_q receptor pathway is present in H9c2 cardiomyoblasts. Vasopressin activates the V_{1a}/G_q-protein/phospholipase-pathway and leads to an increase in intracellular free Ca^{2+} concentration, resulting in hypertrophy of the H9c2 cardiomyoblasts. Since ALC-1 is reexpressed in the hypertrophied ventricle and since it has been shown that in particular Ca^{2+}-dependent signaling pathways play an important role in the development of cardiomyocyte hypertrophy, the aim of the study was to unravel the intercorrelation between Ca^{2+}-calmodulin- and PKC-activated pathways and ALC-1 expression under hypertrophic conditions. In order to elucidate the factors regulating the activity of the hALC-1 promoter, H9c2 cardiomyoblasts were stably transfected with a construct consisting of the luciferase reporter gene under the control of the hALC-1 promoter. A luciferase assay served as a read out system for analysis of hALC-1 promoter activity. Vasopressin was chosen as the stimulus for the induction of hypertrophy in the stably transfected H9c2 cardiomyoblasts.