CHAPTER 1
PATHOGENESIS OF GOUSIEKTE

1.1   Introduction

Livestock losses due to poisoning by the 600-odd toxic plant species in South Africa alone is estimated to number more than 37,000 head of cattle and 250,000 small stock each year (Kellerman *et al*. 1996). Therefore, the prevention of intoxication by plants remains relevant for commercial and rural farmers (Kellerman *et al*. 2005).

Gousiekte ("quick disease") is rated one of the six most important plant poisonings of livestock in southern Africa and causes the death of about 7,000 head of livestock annually (Kellerman *et al*. 1996, Kellerman *et al*. 2005). It is a disease of ruminants characterized by acute heart failure, without any premonitory signs, four to eight weeks after the initial ingestion of certain rubiaceous plants (Kellerman *et al*., 2005). Six plant species have been implicated thus far, namely *Pachystigma pygmaeum* (Fig. 1.1), *Pachystigma thamnus*, *Pachystigma latifolium*, *Pavetta schumanniana* (Fig. 1.2), *Pavetta harborii* (Fig. 1.3) and *Fadogia homblei* (Fig. 1.4). In South Africa, gousiekte occurs in Gauteng, North-West, Limpopo, Mpumalanga and KwaZulu-Natal (Fig. 1.5-1.8). However, gousiekte-inducing plants are also distributed in Botswana, Zimbabwe, Namibia and Mozambique.
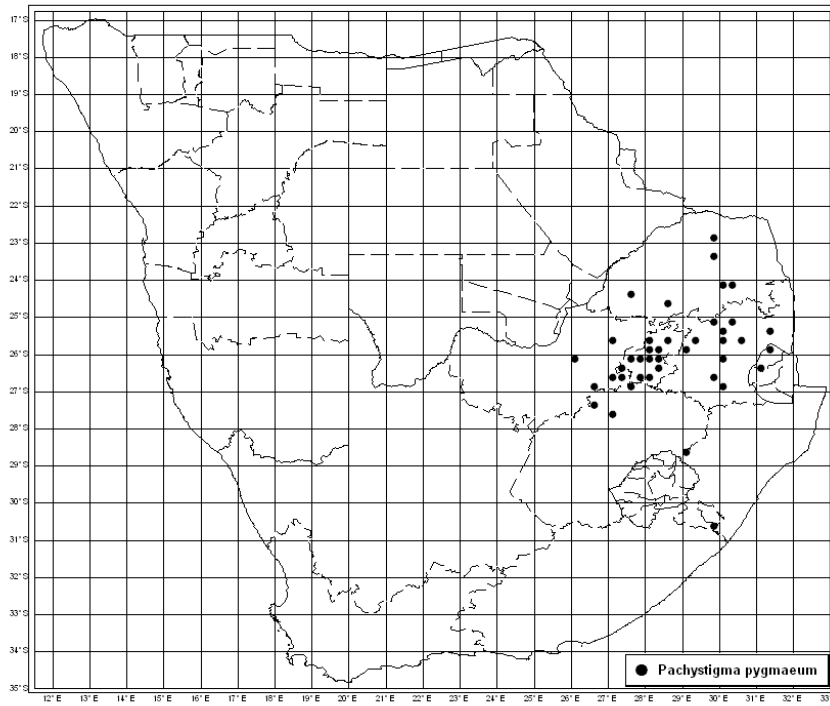

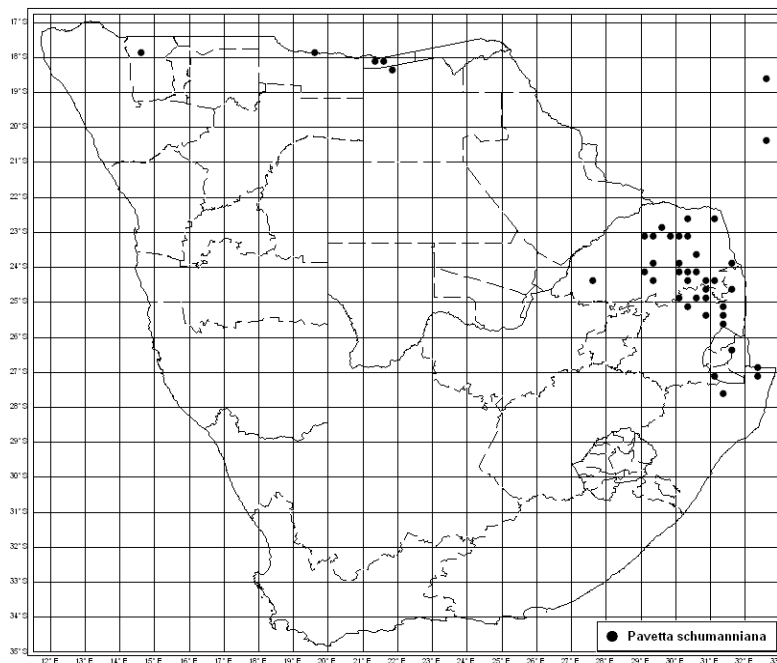**Figure 1.1** *Pachystigma pygmaeum.*

Figure 1.2 *Pavetta schumanniana.*

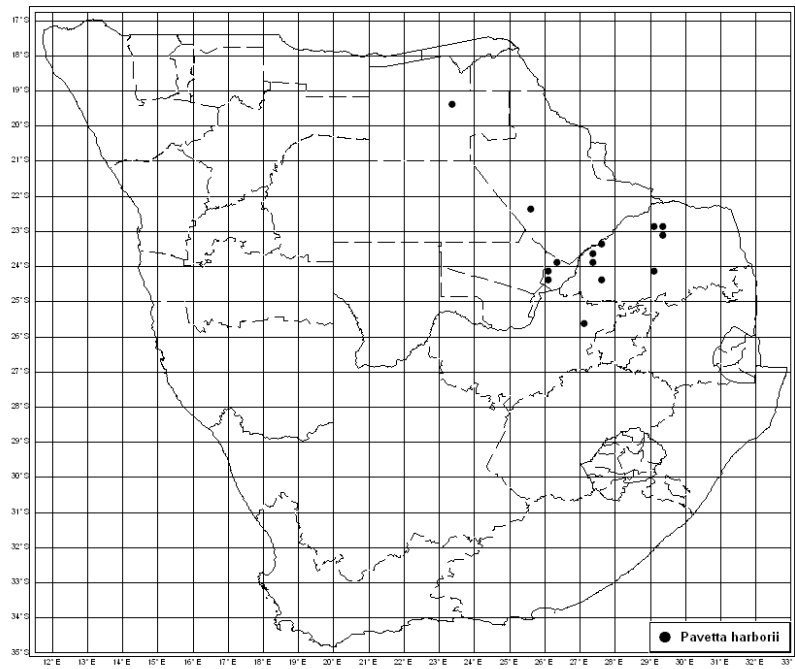**Figure 1.3** *Pavetta harborii.*

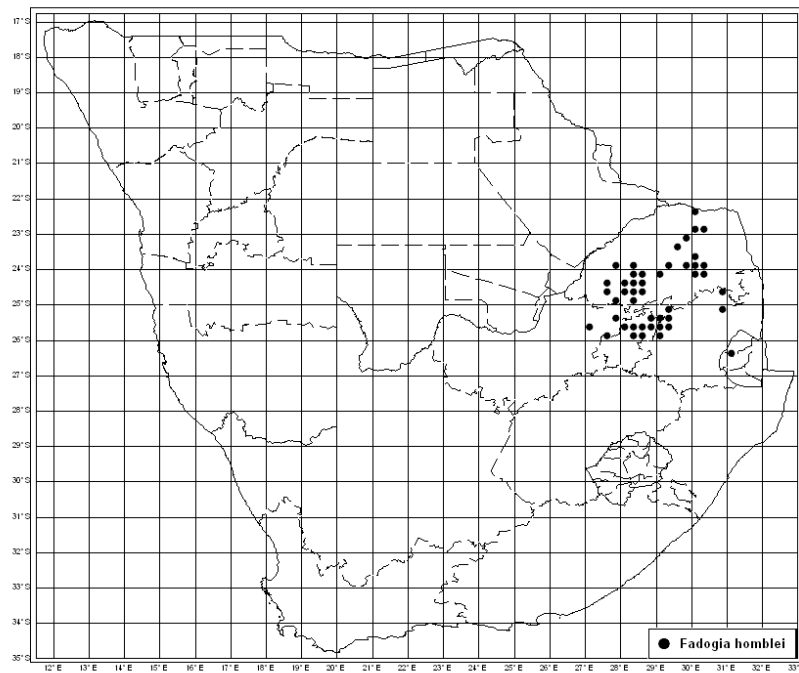**Figure 1.4** *Fadogia homblei.*

Figure 1.5 Distribution of *Pachystigma pygmaeum*.

**Figure 1.6** Distribution of *Pavetta schumanniana*.

Figure 1.7 Distribution of *Pavetta harborii*.

Figure 1.8 Distribution of *Fadogia homblei*.

The toxin that causes gousiekte was first isolated from *Pavetta harborii* and given the trivial name pavetamine (Fourie *et al*., 1995). The structure of pavetamine was elucidated (Fig 1.9). It belongs to the polyamine group and is similar to spermidine, spermine and putrescine (Bode *et al*., 2010). The structure of spermidine ($C_7H_{19}N_3$) is the closest to that of pavetamine, however, pavetamine has a ten carbon backbone and, in addition, four hydroxyl groups.
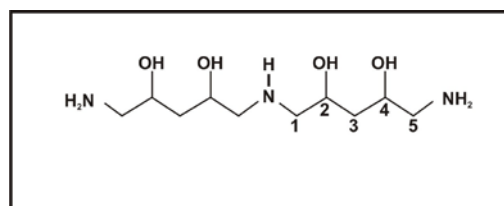

Figure 1.9 Structure of pavetamine

Studies evaluating the clinical pathology parameters and cardiodynamics of gousiekte in ruminants revealed that notable changes appeared terminally, i.e. in the last two weeks of intoxication (Pretorius *et al*., 1973a, van der Walt *et al.,* 1977, van der Walt *et al.,* 1981, Fourie *et al*., 1989). Cardiac failure occurs with the following aberrations: systolic murmurs, gallop rhythms, QRS amplitude alterations, bundle branch blocks, dulling of the first heart sound and decreased myocardial contractility (Pretorius *et al.*, 1973a).

Electron micrographs of the myocardium of sheep intoxicated with *Pachystigma pygmaeum*, showed a lack of register in individual and adjacent myofibres (Prozesky, 2008). The myofibres became disintegrated and had a frayed appearance accompanied by replacement fibrosis. There was a significant increase in intercalated disc length due to the development of complex folds (Schutte *et al*., 1984, Kellerman *et al.*, 2005, Prozesky *et al*., 2005). Furthermore, transmission electron microscopy (TEM) of sections of the hearts of sheep dosed with gousiekte-inducing plants, showed abnormalities of the mitochondria and sarcoplasmic reticula. The mitochondria varied in size, number and shape and demonstrated

the formation of concentric cristae as well as rupture of swollen cristae. The sarcoplasmic reticula were also dilated and proliferated. Pretorius and colleagues (1973b) reported a reduced uptake of Ca^{2+} by sarcoplasmic reticula fragments isolated from the hearts of ruminants with gousiekte. The cardiac muscle of sheep with gousiekte had reduced levels of ATP and creatine phosphate (CrP), and reduced oxygen uptake by isolated mitochondria (Snyman *et al*., 1982).
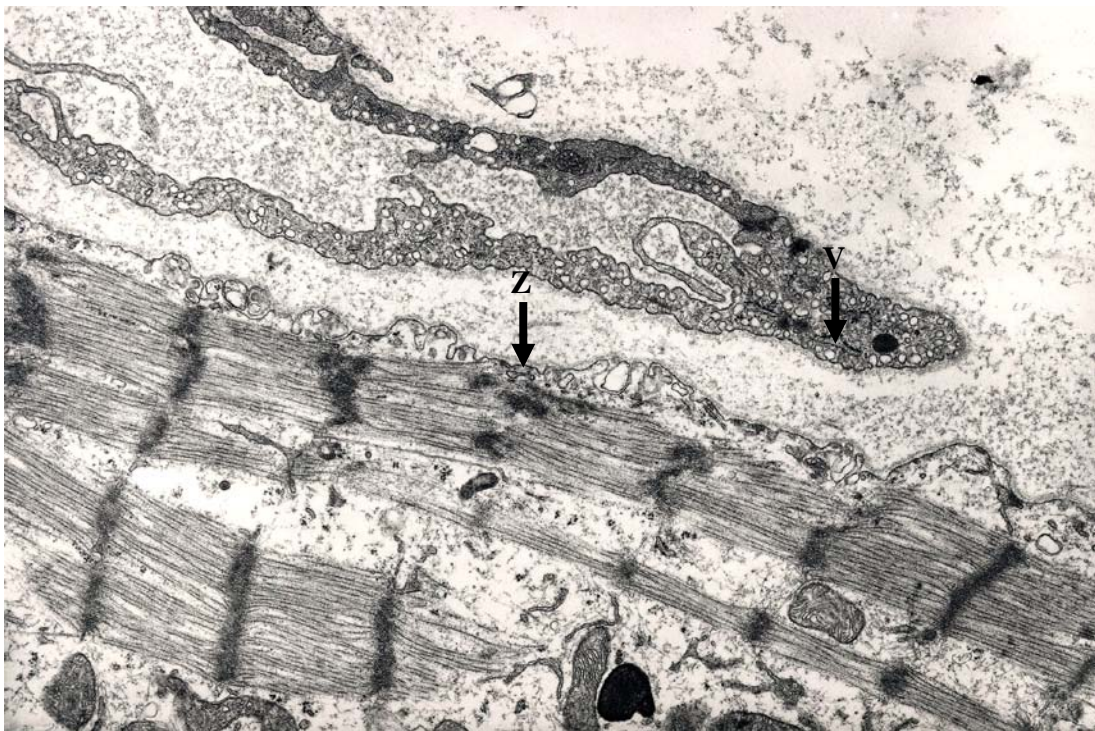
Figure 1.10 Transmission electron micrograph of a gousiekte sheep heart, demonstrating damaged Z-lines and the presence of numerous vacuoles. V, vacuoles that have the appearance of white empty vesicles; Z, disturbed Z-line. (Prozesky *et al*., 2005; Prozesky, 2008).

Rats were affected when extracts prepared from a gousiekte-inducing plant, *Pavetta harborii,* were administered subcutaneously (Hay *et al.,* 2001) or when pavetamine was administered intraperitoneally (Schultz *et al.,* 2001). Several cardiodynamic parameters were determined in rats exposed to *Pavetta harborii* extracts and compared to control rats (Hay *et al.,* 2001).

The contractility (reflected in stroke volume) was reduced by more than 50 % and the cardiac output (product of heart rate and stroke volume) by 40 %. The left ventricle end diastolic pressure (LVEDP) was about seven times higher than the control group, which is indicative of inefficient pumping of blood by the ventricles (Hay *et al*., 2001). Another study was conducted to evaluate whether purified pavetamine had the same cardiodynamic effects
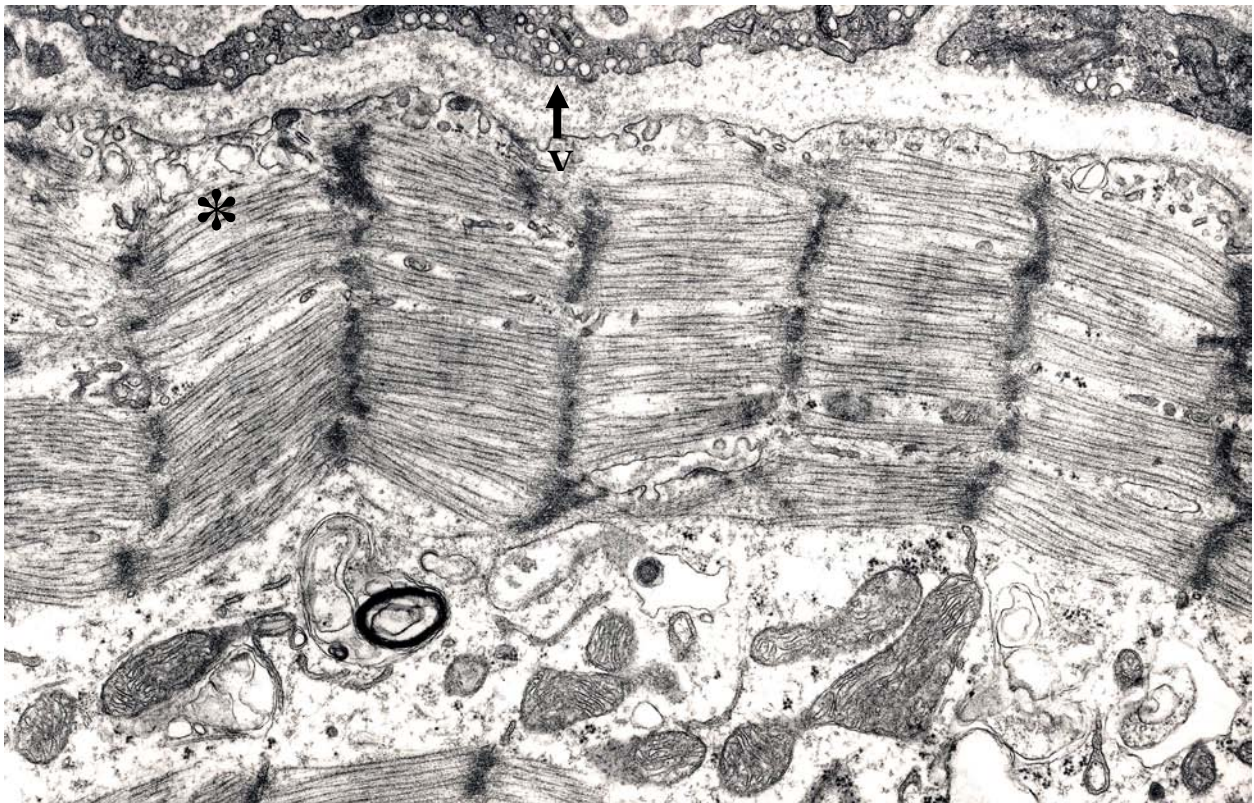
Figure 1.11 Transmission electron micrographs of gousiekte sheep hearts, demonstrating disordered myofibres. V, vacuoles that have the appearance of white empty vesicles; *, disorganized myofibres (Prozesky *et al*., 2005; Prozesky, 2008).
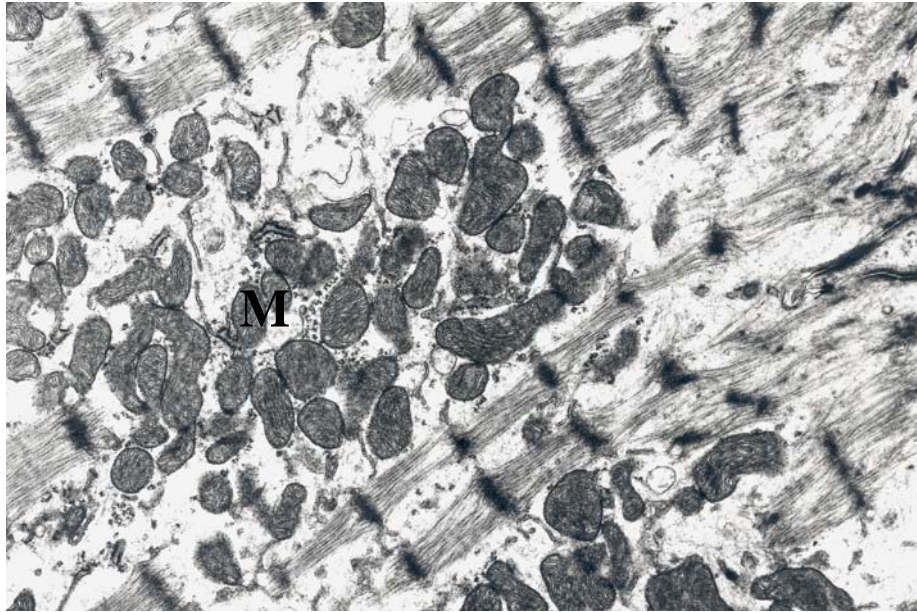
Figure 1.12 Transmission electron micrographs of affected mitochondria (M) in gousiekte sheep hearts. The mitochondria are proliferated and intermingled with the myofibres (Prozesky *et al*., 2005; Prozesky, 2008).
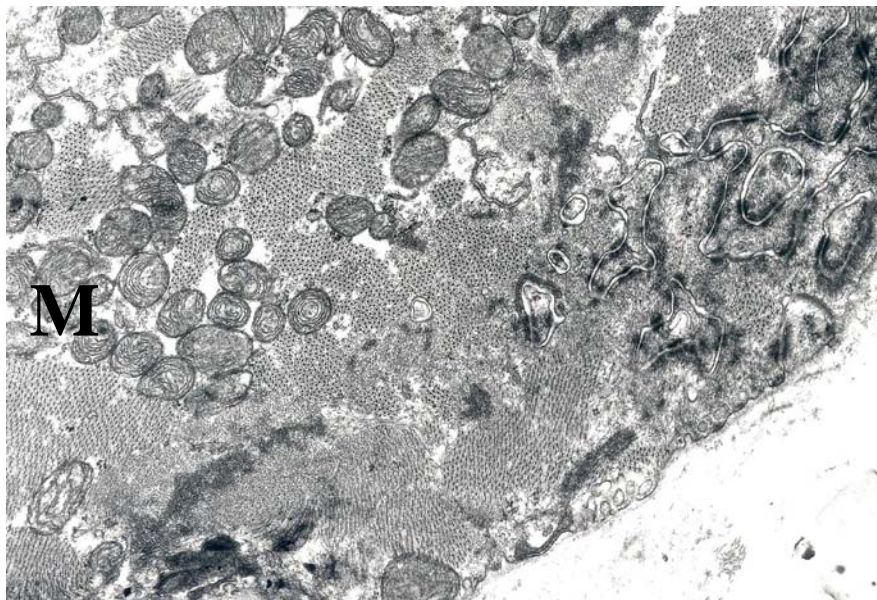
Figure 1.13 Transmission electron micrographs of gousiekte sheep hearts with swollen mitochondrial cristae. M, swollen mitochondrial cristae (Prozesky *et al*., 2005; Prozesky, 2008).

in rats (Hay *et al*., 2008). Pavetamine administered to rats reduced systolic function significantly, but not the diastolic function and heart rate (Hay *et al*., 2008). These authors concluded that the rats were not in an advanced stage of congestive heart failure (high LVEDP and compensatory tachycardia), possibly due to a sub-optimal dose of pavetamine and/or a trial period that was too short.

Schultz and co-workers (2001) investigated the effect of pavetamine on protein synthesis in selected rat tissue. Four hours post exposure of rats to pavetamine, protein synthesis in the heart, liver and kidney was less than 66 %, compared to control rats, but skeletal muscle was not affected. Protein synthesis in the liver and kidney returned to base line levels 24 to 48 h post exposure, while protein synthesis in the heart remained suppressed at 48 h after exposure to the toxin. The authors speculated that depending on the half-life of the cardiac protein, a point will be reached where breakdown of tissues exceeds synthesis, resulting in functional disturbances and abnormal intracellular proteins and organelles.

CHAPTER 2
LITERATURE REVIEW

During the foetal and early perinatal phase, cardiac cells divide by mitosis (Rumyantsev, 1977). However, mature cardiomyocytes are terminally differentiated cells and are not able to proliferate, due to their exit from the cell cycle (Tam *et al*., 1995). In the failing heart, pressure and volume overload leads to cardiac hypertrophy, through activation of a multigene programme in cardiomyocytes. This is accompanied by biogenesis of mitochondria and synthesis of proteins resulting in enlargement of the cardiomyocytes (Morgan *et al*., 1987; Lorell & Carabello, 2000; Hannan, *et al*., 2003). In addition, the efficient functioning of cardiac muscle is dependent upon the proper alignment of myofibrils, microtubules, and intermediate filaments (Gregorio *et al*., 2005).

2.1   Components of the cardiomyocytes

2.1.1   Myofibrillar contractile proteins

A muscle fibre contains myofibrils and is divided into contractile units *viz*. sarcomeres. Each sarcomere contains amongst other proteins, thick and thin filaments, and titin (Fig. 2.1).

2.1.1.1 Titin

Titin, previously called connectin, is a giant macromolecule of 3.0-3.7 mDa, and it spans the length of half a sarcomere to form a third filament system in vertebrate striated muscle. Titin molecules run from the Z-line through the I-band and A-band to the M-band, thereby linking the different sarcomeric regions to one another (Fürst *et al*., 1988). A shorter, stiffer N2B isoform (3.0 mDa) and a longer more compliant N2BA isoform (3.2-3.7 mDa) have been identified (Granzier & Labeit, 2004; Krüger & Linke, 2009). The protein is encoded by one gene with 363 exons, and these different isoforms are the product of alternative gene splicing (Lahmers *et al*., 2004). Decreased myocardial stiffness is often seen in heart failure patients with dilated cardiomyopathy and involves isoform switching of titin (Nagueh *et al*., 2004). The total titin concentration between heart failure patients and controls was the same, but the

N2BA:N2B expression ratio was significantly increased in the heart failure group, which showed significantly lower diastolic stiffness (Nagueh *et al*., 2004).

Titin in the I-band exhibits elastic behaviour upon sarcomeric stretch, contributing to the passive tension of cardiac muscle (Fig. 2.1). The extensible I-band region of titin has multiple segments: the tandemly arranged Ig (immunoglobulin-like) segments, N2B, N2A and the PEVK region, so called because it is rich in proline (P), glutamate (E), valine (V) and lysine (K) (Labeit & Kolmerer, 1995). The PEVK region of titin binds to actin, a reaction which is Ca^{2+}-dependent, involving the S100 Ca^{2+} binding protein A1 (S100A1) (Yamasaki *et al*., 2001). The COOH-terminus of titin is cross-linked to the myosin heavy chain (MHC) and with the myosin-binding protein of the M-band, myomesin (Agarkova & Perriard, 1995). The NH_2-terminus of titin is integrated through telethonin (T-cap) and α-actinin, its Z-line ligands (Gregorio *et al*., 1998). α-Actinin interacts with a number of proteins, two of which are titin and nebulette (Pyle & Solaro, 2004). The C-terminus of titin attaches to the M-line of the sarcomere. The Ig-domain, 14, just inside the I-band titin region, interacts with calpain 1 (Coulis *et al*., 2008). Communication between titin and Z-line proteins provide a mechanism for the cardiac myocyte to sense strain. Multiple phosphorylation sites reside on titin and thus play a role in signalling cascades. The C-terminus domain of titin has protein kinase activity (Yamasaki *et al*., 2002). The titin kinase domain is activated by phosphorylation of tyrosine residues, with subsequent binding of Ca^{2+}/calmodulin (Mayans *et al*., 1998).

The cardiac-specific N2B in the I-band binds two isoforms of the four-and-a half-LIM-domain proteins, FHL1 and FHL2 (Lange *et al*., 2002; Sheikh *et al*., 2008). These proteins act as transcriptional co-activators in the nucleus (Scholl *et al*., 2000) and interact with mitogen-activated protein kinases (MAPKs), especially the extra-cellular-signal-regulated kinase-2 (ERK2) (Sheikh, 2008). The N2A domain in the I-band binds to muscle ankyrin-repeat proteins (MARPs), cardiac ankyrin-repeat protein (CARP), diabetes-related ankyrin-repeat protein and ankyrin-repeat domain protein-2 (Miller *et al*., 2003). These MARPS shuttle between their I-band location and the nucleus, where they act as transcriptional regulators (Kojic *et al*., 2004). Titin also binds to the small ankyrin-1, a protein of the sarcoplasmic reticulum (SR) membrane to position the SR near the Z-line region (Kontrogianni-Konstantopoulos & Bloch, 2003).

2.1.1.2 Myosin

Myosin, the thick filament of the contractile apparatus, is composed of two myosin heavy chains (MHC) and two pairs of light chains, the essential light chain and the regulatory light chain. In the heart, two varieties of the light chains are expressed: the atrial and the ventricular light chains. The portion of MHC closest to the N-terminus is called the head or motor domain and hydrolyses ATP (Palmer, 2005). Myosin head portions cross-bridge with the actin filament in the sarcomere to promote movement during contraction.
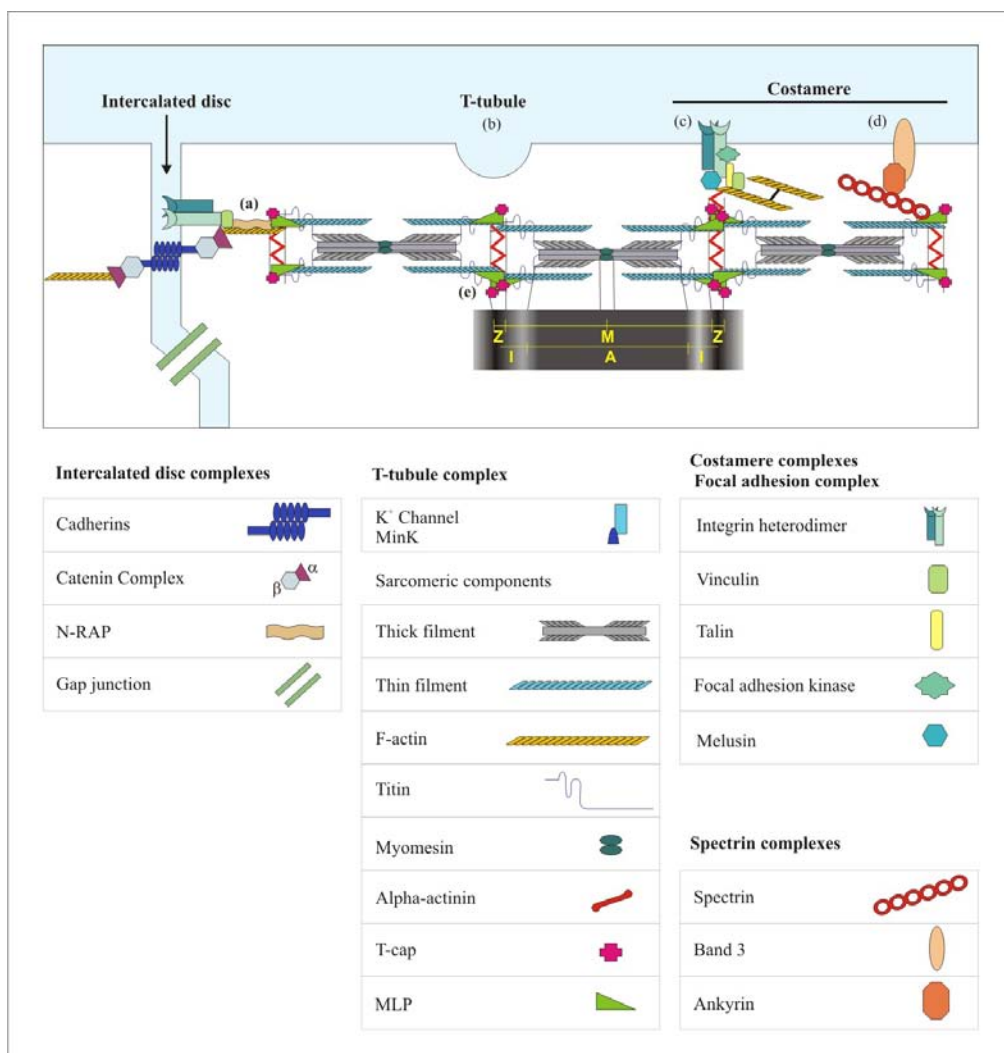

Figure 2.1 Composition of the contractile machinery in the heart (Miller *et al*., 2004). The thick filament is myosin and the thin filament is actin.

Two isoforms exist for myosin heavy chains namely the α-MHC and β-MHC (Xie *et al*., 2003). The thick filament is connected from the M-line to the Z-line by titin and the myosin-binding protein C (MYBP-C) connects myosin with actin (Granzier, *et al.,* 2004).

2.1.1.3 Thin filament (actin) and thin filament regulatory proteins (troponin, tropomyosin)

Actin is one of the most conserved eukaryotic proteins and actin isoforms show greater than 90 % overall sequence homology, except in their 18 N-terminal residues (Lessard, 1988). The main actin in the heart is α-actin. The thin filament proteins tropomyosin (TPM) and the globular Ca^{2+}-binding troponins (TNC) regulate the interaction between actin and the myosin head (Ebashi & Ebashi, 1964) (Fig. 2.2). Troponin consists of three subunits: TNT, TNI and TNC. TNC functions as a Ca^{2+} receptor, TNI (the inhibitory subunit) inhibits actomyosin ATPase and binds actin (Xing *et al*., 2008), and TNT links the entire troponin complex to tropomyosin (TPM) (Greaser & Gergely, 1971). Phosphorylation plays an important part in the regulation of thin filaments. Multiple phosphorylation sites exist on TNI and TNT that affects maximum Ca^{2+} activation, kinetics of the cross-bridge cycle and sensitivity to Ca^{2+}, pH and sarcomere length (Solaro 2001; Wolska *et al*., 2001; Konhilas *et al*., 2003). The level of phosphorylation of TNI and TNT is determined by the following kinases: protein kinase A (PKA), protein kinase C (PKC), protein kinase D (PKD), protein kinase G (PKG), p21-activated kinase (PAK1) and Rho-dependent kinase (ROCK) (Vahebi *et al*., 2005). PAK1 isoform activates protein phosphatase 2A (PP2A) and is a major phosphatase in the heart (Ke *et al*., 2004). Thin filaments also interact with proteins in the Z-line network, which connects to the cytoskeleton and nucleus (Vahebi *et al*., 2005). The barbed ends of the thin filaments are linked to the Z-line through α-actinin and CapZ (Hart & Cooper, 1999).
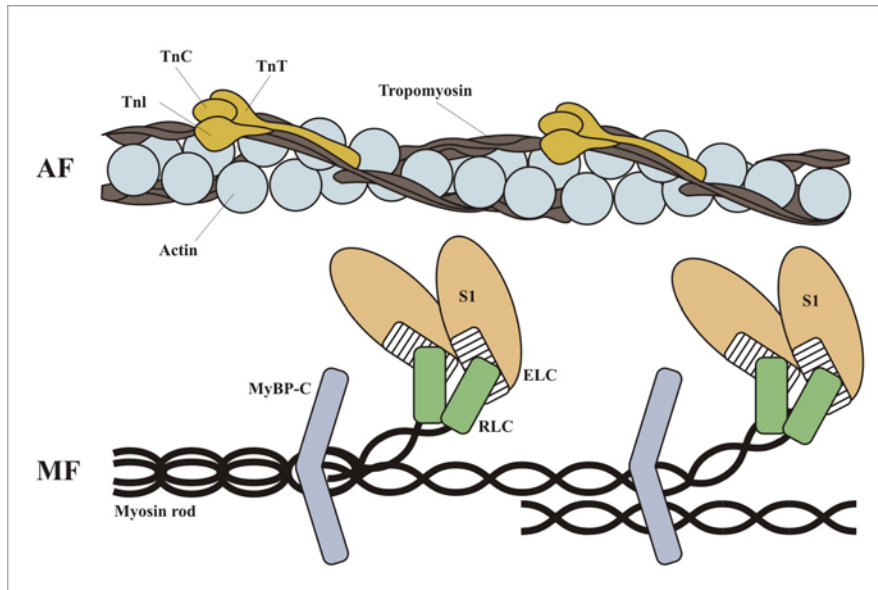
Figure 2.2 The troponin complex. AF: thin actin filament, MF: thick myosin filament, TNI: inhibitory troponin, TNC: Ca^{2+}-binding troponin, TNT: tropomyosin-binding troponin, S1: myosin subfragment-1 (myosin head portion), ELC: essential myosin light chain, RLC: regulatory myosin light chain and MyBP-C: hypothetical localization of the myosin binding protein-C. The M-line is to the left and the Z-line is to the right (Schaub *et al*., 1998).

2.1.2   Z-disc complex

Z-lines cross-link the myofilaments and have a unique position at the interface of the sarcomere, the cytoskeleton, the SR and the sarcolemma (Pyle & Solaro, 2004). The Z-line proteins anchor actin, titin and nebulin filaments. Z-lines are responsible for force transmission, signal transduction and nuclear translocation (Knöll, *et al*., 2002). The following proteins are located at the Z-line: α-actinin (ACTN), muscle LIM protein (MLP), four and a half LIM domain proteins (FHL), enigma factor, actinin-associated LIM protein, FATZ family, myopalladin, telethonin (titin cap or T-cap) and muscle-specific ring finger protein (MURF) (Knöll, *et al*., 2002) (Fig. 2.3). The FATZ family is an acronym for filamin, alpha-actinin and telethonin-binding protein of the Z-disc. The barbed end of actin filaments is capped by CapZ, to anchor sarcomeric actin to the Z-line (Hart & Cooper, 1999).

α-Actinin cross-links sarcomeric actin and plays a role in reversing the polarity of the actins on either side of the Z-line. α-Actinin is a member of the dystrophin superfamily and is capable of cross-linking actin and titin filaments from neighbouring sarcomeres (Djinovic-Carugo *et al.*, 1999). ACTN2 is the isoform present in cardiac cells. α-Actinin is critical in stabilizing the cytoskeleton when contraction begins (Fyrberg *et al.*, 1998).

Nebulette, the cardiac homologue of skeletal muscle protein nebulin, also resides in the Z-line and is associated with the thin filaments (Moncman & Wang, 1999). Nebulette is composed of four domains: an acidic N-terminal domain, a large central repeat domain (nebulin modules), a terminal linker and a SH3 domain (Moncman & Wang, 2002). The SH3 domain is a Src homology-3 (SH3) domain, a family of small globular domains of 60 amino acids and serves as a mediator of protein-protein interactions in signalling pathways (Mayer, 2001) The linker and SH3 domain interact with a number of Z-line associated proteins, namely CapZ, Z-line titin, α-actinin and myopalladin (Bonzo *et al.*, 2008). The nebulin modules of the repeat domain interact with tropomyosin, actin, filamin C and desmin (Bonzo *et al.*, 2008). Myopalladin acts as a scaffold to regulate the actin cytoskeleton (Otey *et al.*, 2005).

Muscle LIM protein (MLP) belongs to a superfamily of proteins that have one or several LIM domains, which are characterized by a cysteine-rich consensus domain. The four and a half LIM domain proteins (FHL) behave as transcriptional co-activators and enhance the transcriptional activity of the androgen receptor (Müller *et al.*, 2000). The enigma family of proteins contain an amino-terminal PDZ domain and one to three carboxy-terminal LIM domains (Guy *et al.*, 1999). Actinin-associated LIM protein is concentrated at the intercalated disc, which forms the junction between neighbouring cardiomyocytes (McKoy *et al.*, 2000). It co-localizes with vinculin, desmin, α-actinin and γ-catenin. Actinin-associated LIM proteins are in part responsible for sarcomeric organization and enhances the ability of α-actinin to cross-link F-actin filaments (Pashmforoush, *et al.*, 2001). Myopalladin is enriched at sites of
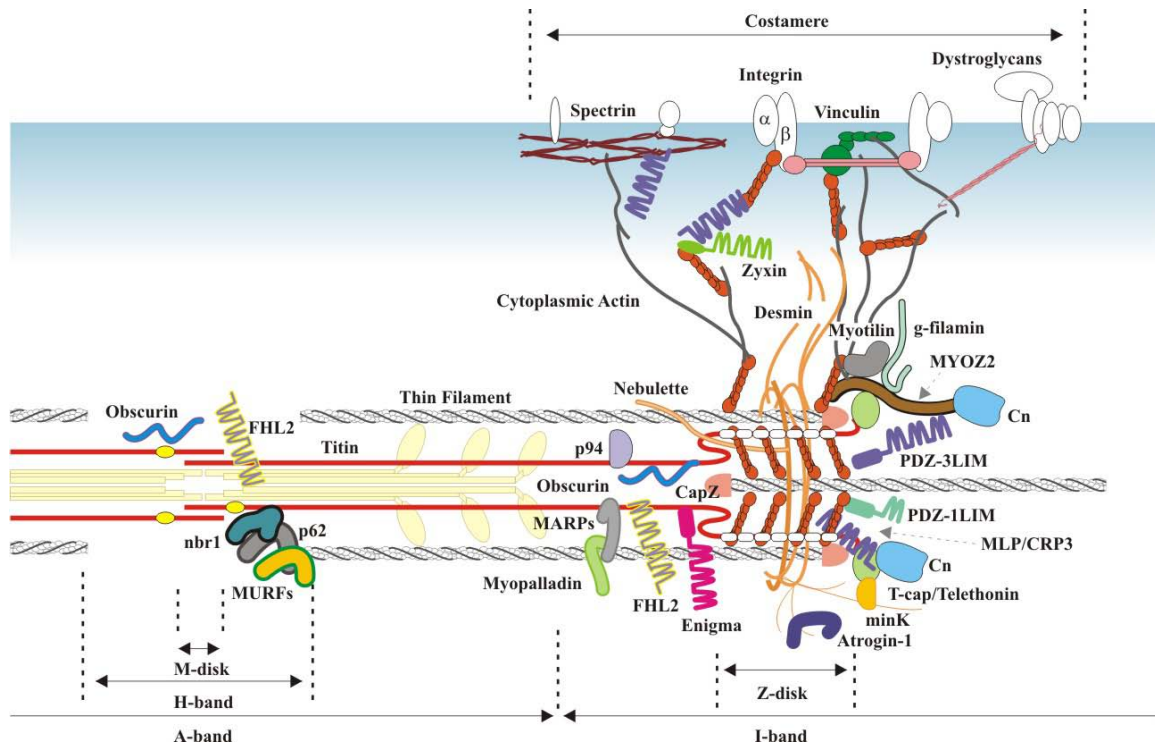
Figure 2.3 Cardiac Z-disc complex. MYOZ2: myozenin 2 (carsarin 1), Cn,: calcineurin, PDZ-3LIM: one-PDZ and three-LIM domain protein, PDZ-1LIM: one-PDZ and one-LIM domain protein, MLP/CRP3: muscle-specific LIM protein/cysteine-rich protein 3, FHL2: four-and-a-half LIM protein 2, MAPRs: muscle ankyrin repeat proteins and MURFs, muscle-specific ring finger proteins (Hoshijima, 2006).

actin filament anchorage (Bang *et al*., 2001). In cardiac cells myopalladin interacts with nebulette via its proline-rich domain and with cardiac ankyrin repeat protein (CARP) via its amino-terminal domain. This interaction suggests a link between myofibrillar organization and gene expression, as CARP is a nuclear protein involved in gene expression (Zou *et al*., 1997). CARP down-regulates expression of cardiac genes for TNC, MLC2 and atrial natriuretic peptide (Jeyaseelan *et al*., 1997).

Telethonin (T-cap or titin cap) anchors titin within Z-lines and is phosphorylated during myofibrillogenesis by titin kinase (Mayans, *et al*., 1998). Telethonin interacts with the potassium channel beta-subunit (minK), suggesting a link in the potassium flux and the Z-line

(Furukawa *et al*., 2001). Muscle-specific ring finger protein 3 (MURF-3) is a cytoskeletal protein that is located at the Z-line and at the M-line (Gregorio *et al*., 2005)

Telethonin, muscle LIM protein, calsarcin-1 and calcineurin can respond in the Z-disc to passive titin-generated tension and belong to the stretch-sensor (Le Winter *et al*., 2007). T-cap is an important link via calcineurin, a phosphatase, between mechanical stretch signals and local Ca^{2+} concentrations. Calsarcin-1 has an inhibitory role on calcineurin (Frey *et al*., 2000). Calcineurin dephosphorylates nuclear factor of activated T-cell, which then enters into the nucleus and promotes transcription (Frey *et al*., 2000). Recently identified proteins residing at the Z-lines include myotilin, S100A1 Ca^{2+}-binding protein and myomaxin (Cox *et al*., 2008). Myotilin, a thin filament-associated protein, binds to F-actin and is responsible for the efficient cross-linking of actin filaments, and prevention of induced disassembly of filaments (Moza *et al*., 2007). Disturbances in the Z-line complex may have catastrophic consequences. These proteins are indirectly involved in the progression of heart failure due to their association with intracellular signalling molecules, protein kinase C (PKC) and calcineurin (Molkentin *et al.,* 1998).

2.1.3   M-band proteins

The M-band is situated in the centre of the A-band, where the thick filaments are interconnected in the middle of the sarcomere (Hornemann *et al*., 2003). Its function is to provide physical stability between thick filaments during contraction. The components of the M-band are myosin, a 185 kDa myomesin, a 165 kDa M-protein and the C-terminal region of titin (Obermann *et al*., 1996). Myomesin is the principal cross-linking protein of the thick filament, a role similar to α-actinin in the Z-line. Myomesin is expressed at a fixed ratio to myosin (Agarkova *et al*., 2004). The myomesin-related M-protein is only present in fast skeletal muscle fibres and cardiomyocytes (Hornemann *et al*., 2003). Muscle-type creatine kinase is bound to the M-band and is an intramyofibrillar ATP-regenerating system for the actin-activated myosin ATPase located nearby on both sides of the M-band (Wallimann *et al*., 1992; Hornemann *et al*., 2003). Spectrin, ankyrin and obscurin might be involved in the lateral connection of M-bands to the sarcolemma (Bagnato *et al*., 2003). The affinity of

myomesin to titin is regulated by phosphorylation and interaction with myofibrillogenesis regulator-1 (Obermann *et al*., 1997; Li *et al*., 2004). Obscurin senses the stress between the myofibrils and the sarcolemma, which activates Ca^{2+}-mediated and Rho GTPase-regulated signalling in the sarcomere (Young *et al*., 2001).
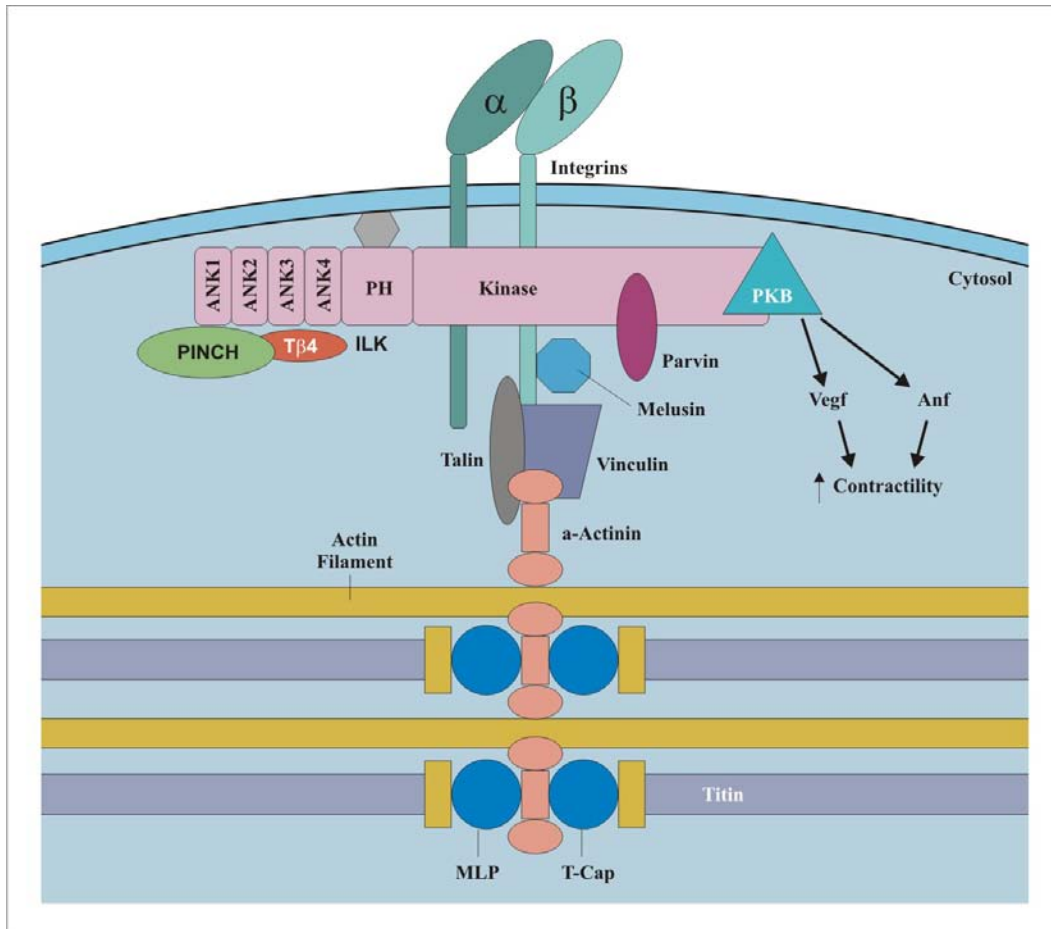
2.1.4   Costameres

Between the extracellular matrix, the sarcolemma and the Z-disc, the costameres form a centre of communication (Ervasti, 2003) (Fig. 2.4a). Costameres transmit contractile force from the myofibrils across the sarcolemma to the extracellular matrix and maintain the alignment of the myofibrils, a prerequisite for contraction (Quach & Rando, 2006). Costameres are rich in the focal adhesion proteins vinculin, integrins, talin, paxillin and Crk-associated substrate (Cas) (Quach & Rando, 2006) (Fig. 2.4b). Vinculin transmits stretch signals from the sarcolemma to actin/α-actinin in the sarcomere (Heling *et al*., 2000). Talin and vinculin link the cytoplasmic domains of integrins to the Z-disc (Heling *et al*., 2000). Integrins connect components of the extracellular matrix with the actin cytoskeleton (Cox *et al*., 2008). Integrins interact with adaptor proteins like filamin, α-actinin, tensin and talin (Bershadsky *et al*., 2003). Integrins also interact with signalling proteins such as focal adhesion kinase (FAK), src-family tyrosine kinases, melusin, integrin-linked kinase and small GTPases (Ervasti, 2003). Focal adhesion kinase plays an essential role in integrin-mediated signal transduction (Cox *et al.,* 2008). Melusin, another signalling protein, is located at the costameres near the Z-disc, where it binds to the intracytoplasmic tail of β1-integrin (Brancaccio *et al*., 1999). Integrin-linked kinase directly interacts with β1-integrin (Hannigan *et al*., 1996). Paxillin binds to many proteins involved in the organization of the actin cytoskeleton (Turner, 2000). FAK, Cas and paxillin are localized in the sarcomeric Z-line (Kovacic-Milivojević *et al*., 2001). Laminin-2, collagens and fibronectin, all of which are extracellular matrix proteins, bind to specific integrins and align the costameres (Quach & Rando, 2006).
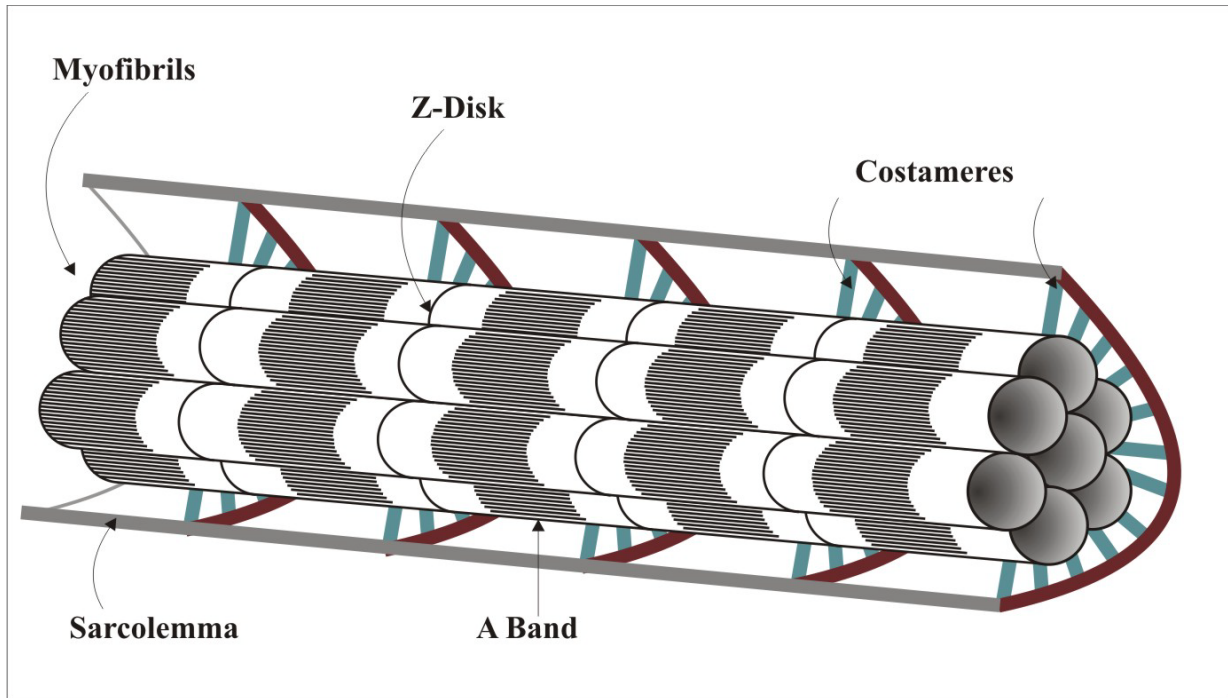
Figure 2.4a Structure of costamere and Z-disc (Ervasti, 2003).

2.1.5 Intercalated discs

The intercalated discs (IDs) between individual cardiomyocytes ensure mechanical coupling and propagation of electrical impulses throughout the heart (Fig. 2.5) (Noorman *et al*., 2009). The IDs consist of three protein complexes: the adherens junctions (AJs) (Fig. 2.5a), desmosomal junctions (Fig. 2.5b) and gap junctions (GJs) (Fig. 2.5c). The adherens junctions (AJs) are unique to cardiac cells in that they connect the cardiomyocytes with each other at the intercalated discs (ID), as well as the conductive Purkinje fibre cells (Franke *et al*., 2006). The AJs mechanically link the cardiomyocytes with the actin cytoskeleton (Niessen, 2007). AJs are also the anchor-point for cardiomyocyte attachment, ensuring transmission of contractile force from cell to cell. Cadherins are transmembrane proteins that form complexes with cytosolic α-, β-, γ-plakoglobin and p120 catenin, thereby establishing the connection to the actin cytoskeleton (Niessen, 2007; Noorman *et al*., 2009).

Figure 2.4b Components of the costameres (Srivastava & Yu., 2006). ANF, atrial natriuretic factor; ANK1, ankyrin; ILK, integrin-linked kinase; MLP, muscle LIM domain protein; PH, plekstrin homology domain of ILK; PINCH, particularly interesting Cys-His-rich protein; PKB, protein kinase B; PKC, protein kinase C; T-CAP, thelethonin of titin cap; Tβ4, thymosin β4; VEGF, vascular endothelial growth factor

The desmosomes or desmosomal junctions provide support between myocytes via their interaction with the intermediate filament (IF) cytoskeleton (Noorman, *et. al*., 2009). Desmoplakin, plakoglobin (γ-catenin) and plakophilin 2 (in the intracellular environment) mediate the linkage between the IF and the desmosomal catherins, desmocollin and desmoglein, in the intercellular part of the cell (Garrod & Chidgey, 2008). The desmosomes give mechanical strength to tissues because they form adhesive bonds in a network.

The gap junctions (GJs) mediate direct communication between adjacent cells (Noorman *et al.*, 2009). Passive diffusion of various compounds, metabolites, water and ions up to a mass of 1000 Da occur through these intercellular ion channels, that links the cytoplasm of neighbouring cells (Elfgang *et al.*, 1995). GJ channels consist of twelve connexin subunits, six of which are contributed by each cell. These six connexion subunits form a hemi-channel in the sarcolemma (Noorman *et al.*, 2009). Connexin43 is the most important isoform in the ventricular myocardium (Beyer *et al.*, 1987). A common feature during cardiac remodelling and heart failure is changes in GJ expression and distribution, with levels of connexin43 reduced and migration from the ID to the lateral sides of the cell (Noorman *et al.*, 2009). Molecules at the intercalated disc also serve as mechanical stress sensors (Hoshijima, 2006). One molecule to perform this function is nebulin-related anchoring protein, which binds muscle LIM protein (MLP) (Ehler *et al.*, 2001), actin, vinculin and talin (Luo *et al.*, 1999).
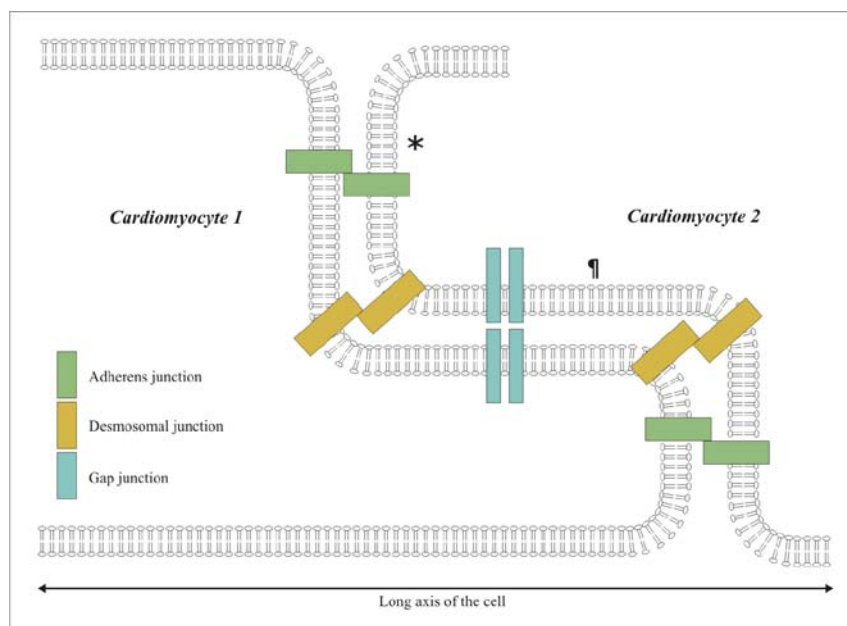
Figure 2.5a The intercalated discs consist of the adherens junctions, desmosomes and the gap junctions (Noorman *et al.*, 2009).
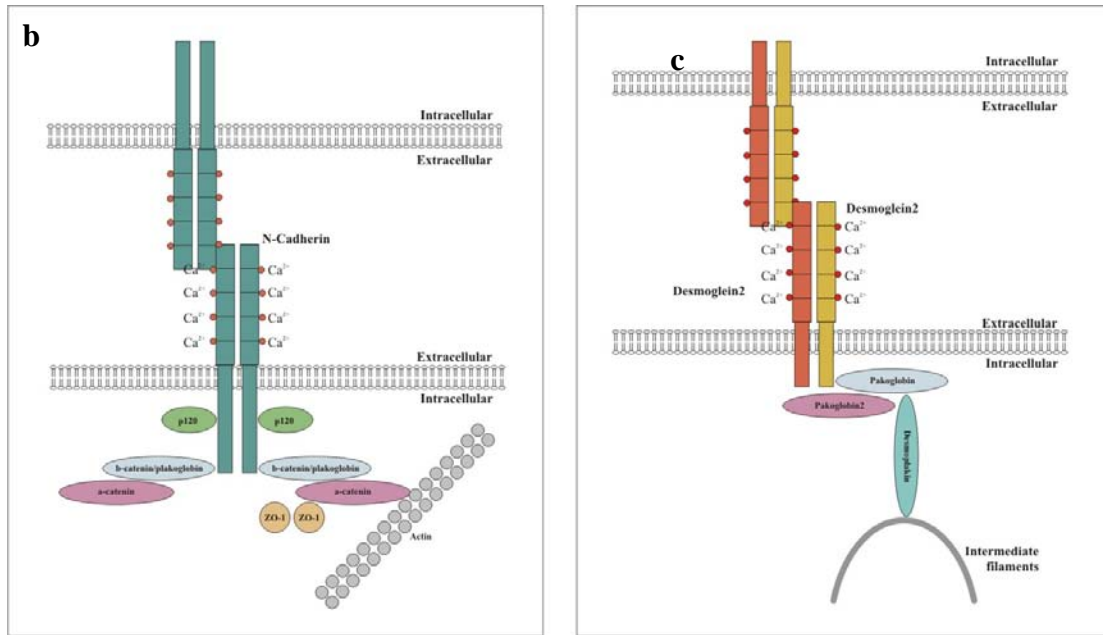
Figure 2.5b Adherens junctions connect adjoining cells to each other through N-cadherin (Noorman *et al*., 2009). The adherens junction protein α-catenin binds to the actin cytoskeleton. ZO-1, scaffolding protein zonula occludens-1. **Figure 2.5c** Desmosomes connect neighbouring cells to each other. The extracellular part consists of two desmosomal cadherins: desmoglein-2 and desmocollin-2. The cadherins are linked to the intermediate filaments.
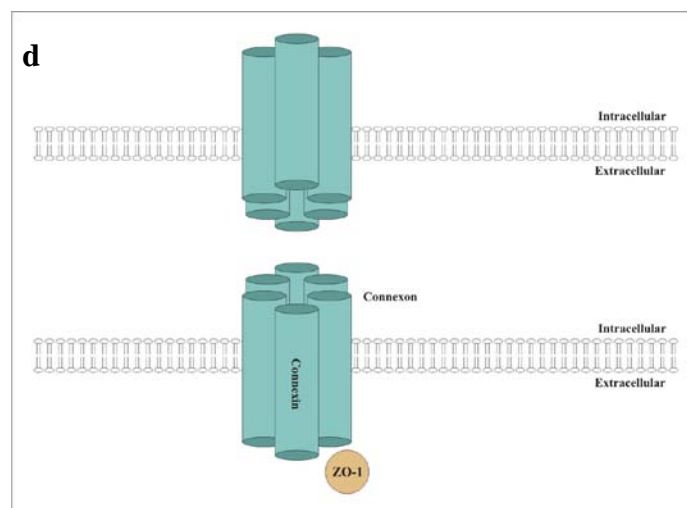
Figure 2.5d Gap junctions consist of two connexons, one of each delivered by each cell (Noorman *et al*., 2009).

2.1.6   Cardiac extramyofibrillar cytoskeleton proteins: F-actin, microtubules and intermediate filaments

The cytoskeleton gives mechanical support to the cell and mediates cell motility, organelle movement, cytokinesis, muscle contraction and plays a role in protein synthesis, intracellular trafficking and organelle transport within the cell (Rogers & Gelfand, 2000). The cytoskeleton proteins include the microfilaments (actins), microtubules (tubulins) and the intermediate filaments (desmin).

2.1.6.1 F-actin

Filamentous actin (F-actin) is formed by the assembly of monomeric actin, also called globular-actin (G-actin) (Fig. 2.6). G-actin is polar, thus F-actin is also polar with a barbed end (plus end) and a pointed end (minus end) (Kustermans *et al*., 2008). F-actin is organized into complex structures. Actin filaments can be arranged in parallel as in filopodia or organized into orthogonal, net-like meshworks as in lamellipodia (Revenu *et al*., 2004). Anti-parallel actin filaments are found in stress-fibres. Actin monomers bind ATP and ADP. After incorporation of monomeric actin into a filament, the enzymatic activity of actin will in turn hydrolyze the bound ATP to ADP and Pi. The dynamics of actin (the coordinated assembly and disassembly of actin filaments in response to cellular signalling) are regulated by actin-binding proteins. Capping proteins regulate the length of actin filaments by either stabilizing an actin filament or promoting disassembly. Two compounds, phalloidin and jasplakinolide, favour the polymerization of actin, while cytochalasin D (Schliwa, 1982) and latrunculin B (Spector *et al*., 1983) inhibit actin polymerization.

The small Rho GTPases (RHO, RAC and CDC42) are actin dynamics-regulating proteins (Jaffe & Hall, 2005). RHO A activation of fibroblasts leads to the formation of actin stress fibres and focal adhesion complexes (Ridley & Hall, 1992). Actin polymerization is facilitated by activation of RAC 1 at the cell periphery to produce lamellipodia and membrane ruffling (Ridley *et al*., 1992). Activation of CDC42 induces filopodia (Nobes & Hall, 1995).
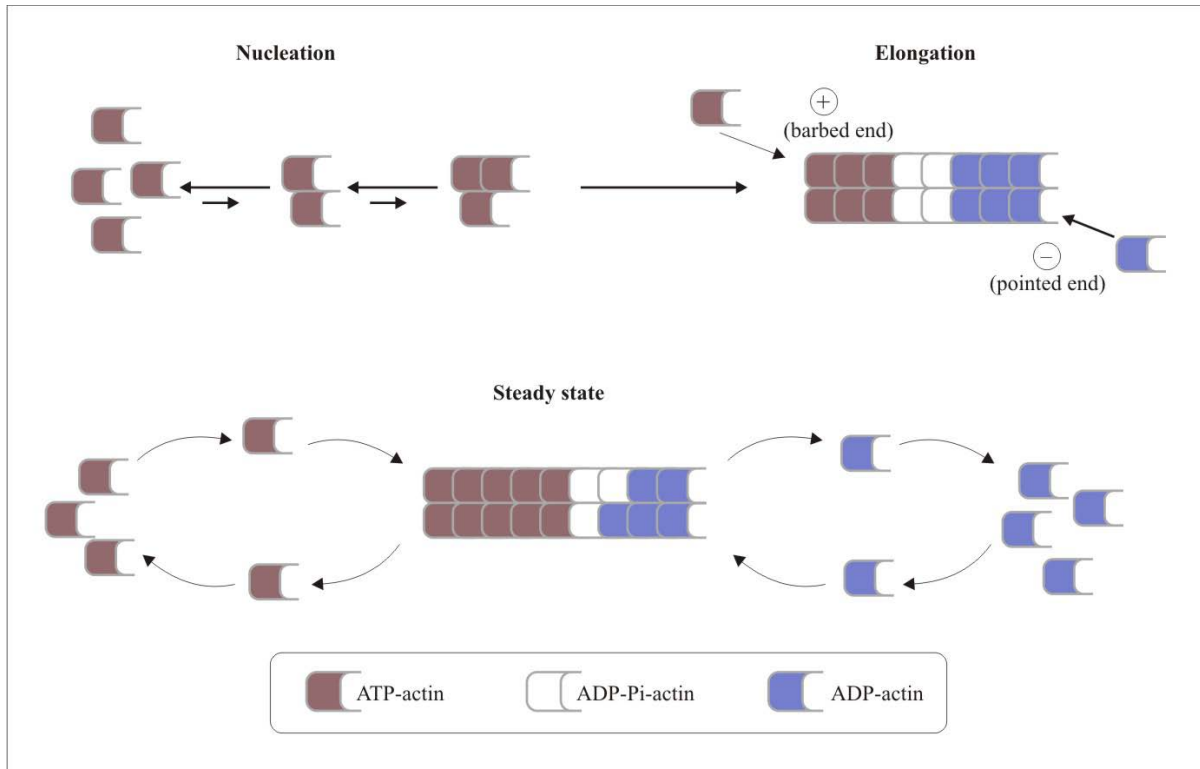
Figure 2.6 Monomeric G-actin is polymerized to form F-actin with a barbed end (plus end) and pointed end (minus end) (Kustermans *et al*., 2008).

Additional functions of the RHO subfamily are their ability to regulate cell polarity, gene transcription and cell cycle progression (Jaffe & Hall, 2005).

G protein-coupled receptors (GPCRs) can activate small GTPases, RHO A and RAC 1 in Swiss 3T3 cells (Ridley & Hall, 1992). Activation of RHO-associated coiled-coil containing protein kinase (ROCK), a downstream mediator of RHO A GTPase, leads to cardiac hypertrophy and remodeling (Kobayashi & Matsuoka, 2002). Activation of ROCK during apoptosis, results in increased myosin activity, bundling of F-actin, actin-myosin contractile force generation and membrane blebbing (Song *et al*., 2002). ROCK acts as a negative regulator of the PI3-kinase/AKT pathway, a pro-survival pathway functioning in endothelial cells during ischemia-reperfusion (Van Der Heijden *et al*., 2008). p21-Activated kinase 1 (PAK1), the predominant isoform in the heart, is activated by the small GTPases CDC42 and RAC-1 (Manser & Lim, 1999). PAK1 is involved in diverse cellular functions, such as cytoskeleton reorganization and proliferation (Sheehan *et al*., 2007). PAK1 forms a signalling

complex with protein phosphatase 2A (PP2A), that modulates the myofilament Ca^{2+} sensitivity and intracellular Ca^{2+} fluxes (Sheehan *et al*., 2007).

The cardiac L-type calcium channels (I_{Ca-L}) are anchored to F-actin by stabilizing proteins that control the activity of these channels (Lader *et al*., 1999). Activation of the L-type Ca^{2+} channels, that involves F-actin, increases the mitochondrial membrane potential ($\Delta\Psi_m$) (Viola *et al*., 2009). Cytoskeletal proteins regulate the subcellular distribution of mitochondria and the L-type Ca^{2+} channels regulate mitochondrial function via the cytoskeleton (Viola, *et al*., 2009).

Disturbances in the structure of F-actin by cold shock reduce protein synthesis in Chinese hamster ovary (CHO) cells (Stapulionist *et al*., 1997). Several authors have demonstrated that F-actin is involved in chromatin remodeling, transcription, RNA processing and nuclear export (Miralles & Visa, 2006; Vartiainen *et al*., 2007; Farrants, 2008; Vartiainen, 2008; Ye *et al*., 2008; Gieni & Hendzel, 2009). Nuclear actin is required for efficient transcription by all three classes of RNA polymerases (Fomproix & Percipalle, 2004; Hofmann *et al*., 2004; Hu *et al*., 2004). NM1, a myosin isoform, is also present in the nucleus and interacts with actin to execute specific nuclear functions (Ye *et al*., 2008). These authors also reported that for efficient transcription to occur, actin must be in the polymeric form, as drugs that inhibit actin polymerization e.g. cytochalasin D and latrunculin B, significantly decreased pre-rRNA synthesis. Serum response factor (SRF), a MADS-box transcription factor, is sensitive to the state of actin polymerization (Kuwahara *et al*., 2005). G-actin inhibits serum response factor (SRF) activity, while polymerization of actin, as a result of serum stimulation and RHO A signalling, stimulates SRF activity (Sotiropoulos *et al.,* 1999).

2.1.6.2 Microtubules

Microtubules of the cardiomyocyte cytoskeleton are involved in protein synthesis, intracellular trafficking and intracellular signalling (Rogers & Gelfand, 2000). This network is dynamic through self-association of α,β-tubulin dimers. Microtubules are in a constant state of depolymerization and repolymerization. This dynamic state and their abundance may

change the stiffness of the cytoskeleton, which influences the contractility of the cardiomyocytes (Ishibashi *et al*., 2003). In pressure-overload cardiac hypertrophy, there is an increase in the microtubule network, which causes the contractile dysfunction (Tsutsui *et al*., 1993). Gómez and colleagues (1999) reported that microtubule depolarization by colchicine increased Ca^{2+} current and SR Ca^{2+} release of excitation-contraction coupling. Thus, besides a mechanical role, the microtubules are important modulators of cardiac function through Ca^{2+} signalling. Microtubules are unbranched tubular structures with their polar plus ends orientated towards the cell periphery and minus ends focused at the perinuclear region (Moss & Lane, 2006).

2.1.6.3 Intermediate filaments (IF)

The intermediate filaments (IF) of muscle cells contain several proteins namely desmin, vimentin, nestin, synemin, syncoilin, lamins and cytokeratins (Carlsson & Thornell, 2001). Of these, desmin is the major muscle-specific IF protein. It is located mainly in the Z-disc of striated muscle and plays an essential role in maintaining the cytoarchitecture as well as connecting the entire sarcomere to the sarcolemma, T-tubules, mitochondria and the nuclei (Conover *et al*., 2009). Skeletal and cardiac muscle of desmin knock-out mice (Des-/-) had misaligned sarcomeres and disintegrated myofibrils, and accumulated mitochondria (Conover *et al*., 2009). The network formed by IFs is involved in functions such as mechanical integration of all contractile actions, cellular integrity, force transmission, mechanical signalling and integration of organelle structure and function (Capetanaki *et al*., 2007). Desmin filaments extend from the Z-discs towards the nuclear pores, leading to *de novo* gene activity (Tolstonog *et al*., 2002). Desmin also plays a significant role in mitochondrial morphology, positioning and respiratory function in cardiac and skeletal muscle (Milner *et al*., 2000).

2.2 The role of mitochondria in the heart

The heart has a high energy demand and reduced energy generation leads to dysregulation of processes critical for cardiac pump function, including Ca^{2+} handling and contractile function.

There is a strong interrelationship between coronary blood flow, myocardial oxygen consumption and contractile performance. Energy metabolism is linked to gene expression, enzyme regulation and contractile function. The immediate response to a decrease in blood flow affects the transfer of substrates for ATP synthesis. Long-chain fatty acids are the major energy source for the heart, which are metabolized to acetyl coenzyme A, which is then metabolized in the Krebs cycle. When the levels of these fatty acids are low, the heart utilizes glucose for oxidative metabolism. Intracellular accumulation of protons, inorganic phosphate, sodium and calcium, is the result of ATP hydrolysis and lactate production during anaerobic energy metabolism (Depre *et al.*, 2006). ATP synthesis is carried out in the mitochondria through oxidative phosphorylation (OXPHOS). Three metabolic processes are involved in ATP production namely glycolysis, the Krebs cycle (also called the citric acid cycle) and the electron transport chain (ETC). Long-chain fatty acid oxidation generates the coenzymes NADH and FADH for entry into the electron transport chain.

2.2.1 Generation of energy in the mitochondria

Complex I (NADH dehydrogenase or NADH:quinone oxidoreductase) is the first enzyme of the mitochondrial electron transport chain. Complex I translocates 4 protons across the inner membrane per molecule of oxidized NADH to coenzyme Q, helping to establish the electrochemical potential used to produce ATP (Hatefi *et al.*, 1959). Rotenone is an inhibitor of complex I activity (Lindahl & Oberg, 1961). Complex II (succinate-ubiquinone oxidoreductase), bound to the inner membrane, catalyzes the oxidation of succinate to fumarate with the reduction of ubiquinone (Q) to ubiquinol (QH_2) (Ziegler & Doeg, 1962). Complex III (ubiquinone-cytochrome *c* oxidoreductase), catalyzes the reduction of cytochrome *c* by oxidation of coenzyme Q (CoQ) and the concomitant pumping of 4 protons from the mitochondrial matrix to the intermembrane space (Green & Burkhard, 1961). Antimycin is an inhibitor of complex III (Alexandre & Lehninger, 1984). Complex IV (cytochrome *c* oxidase) receives an electron from each of four cytochrome *c* molecules, and transfers them to one oxygen molecule, converting molecular oxygen to two molecules of water. In the process, it binds four protons from the inner aqueous phase to produce water, and in addition translocates four protons across the membrane, helping to establish a transmembrane difference of proton electrochemical potential that the ATP synthase then uses

to synthesize ATP (Warburg, 1926). Cyanide is an inhibitor of complex IV (Slater, 1950). Complex V (F1-ATP synthase) synthesizes adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate (Boyer, 2002). This energy is often in the form of protons moving down an electrochemical gradient from the inter-membrane space into the matrix in mitochondria. See Fig. 2.7 for an illustration of the reactions in OXPHOS.

Reactive oxygen species (ROS) are free radicals with one unpaired electron and are derived from molecular oxygen. Superoxide anion (O_2^-) is the precursor of most other ROS. Oxidative stress occurs when there is an imbalance between production and detoxification of ROS. Defects in oxidative phosphorylation (OXPHOS) lead to decreased energy production, as well as increased formation of superoxide, hydrogen peroxide (H_2O_2), peroxynitrite and hydroxyl radicals (Koopman, *et al.*, 2005). Accumulation of ROS results in DNA damage, protein oxidation and lipid peroxidation.

2.2.2   Mitochondrial membrane potential ($\Delta\Psi_m$)

The electrochemical potential is generated by the respiratory chain enzymes in the inner mitochondrial membrane (Fig. 2.7). Energy is provided by transfer of electrons from substrates to oxygen for pumping protons across the membrane and thus generates an electrochemical potential ($\Delta\mu_{H+}$):

$$(\Delta\mu_{H+}) = -2.3RT\Delta pH + F\Delta\Psi_m,$$

where ΔpH is the pH difference, R is the universal gas constant, T is the absolute temperature, F is the Faraday constant and $\Delta\Psi_m$ is the mitochondrial membrane potential

across the mitochondrial membrane, with the negative charge inside the mitochondria (Labajova *et al.*, 2006). The $\Delta\Psi_m$ is expressed in milliVolt units (mV). Lipid-soluble cations and anions are widely used for measurement of the $\Delta\Psi_m$. Popular fluorescent probes include rhodamine 123 and tetramethylrhodamine methyl ester (JC-1).

Apoptosis leads to dissipation or depolarization of the $\Delta\Psi_m$. Valinomycin is a K^+-selective ionophore which uncouples OXPHOS, thereby causing collapse of the $\Delta\Psi_m$ (Furlong *et al.*,

1998). Dissipation (depolarization) of the $\Delta\Psi_m$ leads to autophagic degradation of mitochondria, suggesting that autophagy is a house-keeping function (Twig *et al*., 2008). Mitochondrial autophagy is termed mitophagy (Elmore *et al*., 2001).
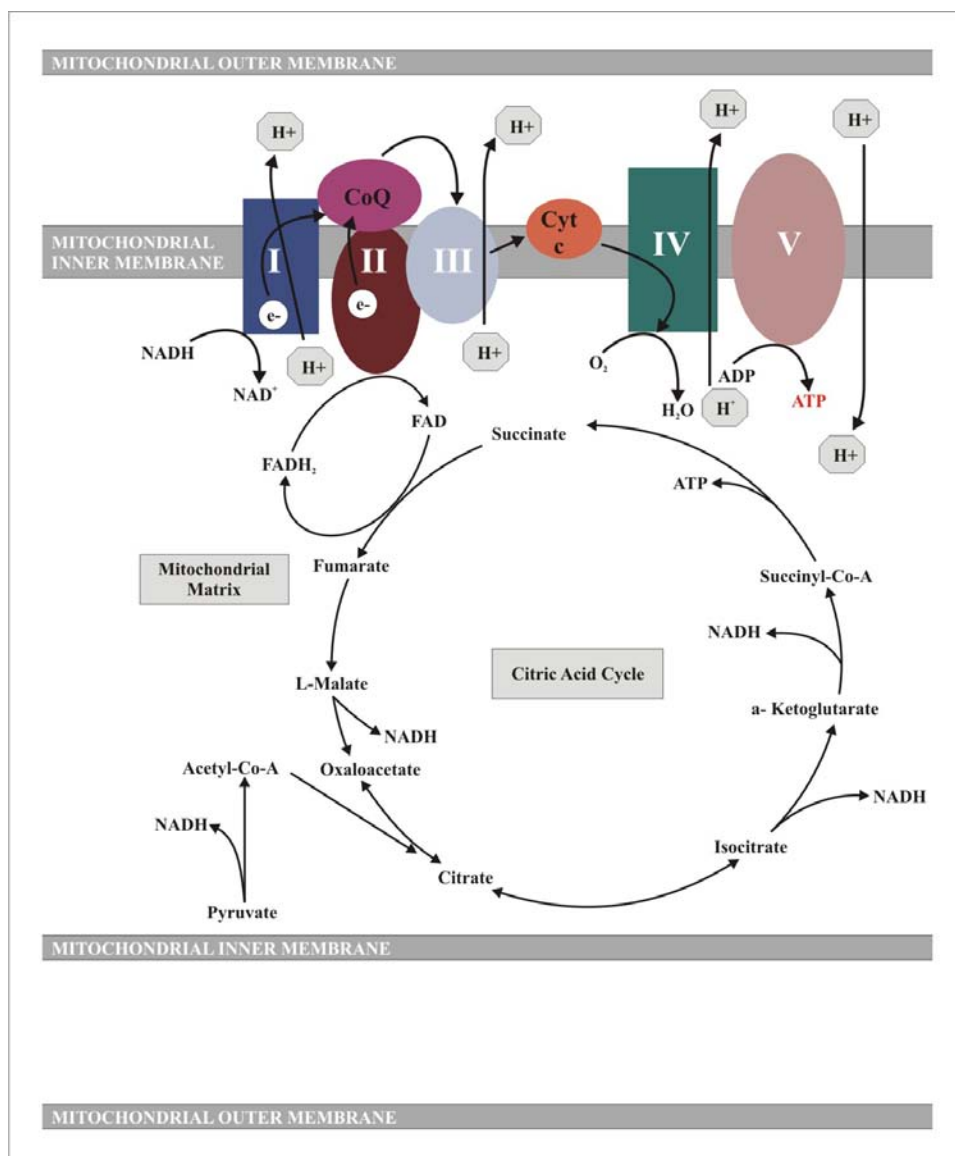
Figure 2.7 Diagrammatic scheme for oxidative phosphorylation in the mitochondria and its link to the citric acid cycle (Cognitive Enhancement Research Institute; http://www.ceri.com/mitobox.htm).

2.2.3 The mitochondrial permeability transition pore (MPTP)

The MPTP is a polyprotein complex of about 600 kDa in size situated between the outer- and inner mitochondrial membrane. It is involved in the regulation of the mitochondrial matrix homeostasis (Clerk *et al*., 2003). Three essential proteins form part of the MPTP *viz.* (1) the adenine nucleotide transporter (ANT), situated in the inner mitochondrial membrane and maintaining the proton gradient required for energy production; (2) Cyclophilin D, known as a mitochondrial peptidyl-prolyl *cis-trans* isomerase and (3) the mitochondrial phosphate carrier (PiC) (Halestrap & Pasdois, 2009). Previously, it was proposed that the voltage activated anion channel was also involved in the MPTP, but was later eliminated as a component of the MPTP (Halestrap & Pasdois, 2009). The MPTP opens with pathological increases in Ca^{2+}, adenine nucleotide depletion, high inorganic phosphate (Pi) and oxidative stress. Opening of the MPTP leads to dissipation of the proton motive force, the pH gradient and the $\Delta\Psi_m$. After opening of the pore, mitochondria can not synthesize ATP via OXPHOS and ATPase activity reverses and starts to break down the ATP, leading to an energy collapse. When the MPTP is opened, mitochondrial swelling and rupture of the outer mitochondrial membrane also occurs, leading to the release of cytochrome *c*. The MPTP is a non-selective pore, permeable to any molecule less than 1.5 kDa. Cyclosporin A (CsA) inhibits opening of the MPTP, where it blocks the association of cyclophilin D and ANT (Crompton *et al.*, 1988; Crompton, 1999).

2.3 Calcium homeostasis

Calcium plays an important role as second messenger in cellular processes such as muscle contraction, secretion, cell division, cell cycle progression, energy production and gene transcription (Maco, *et al.*, 2001). The excitation-contraction coupling of the heart is tightly controlled by the regulated release and uptake of intracellular Ca^{2+} between the SR and the cytoplasm (Fig. 2.8). Contraction is initiated when Ca^{2+} enters the cell via the L-type Ca^{2+} channels (dihydropyridine receptors; DHPRs) in the sarcolemma. This in turn releases a larger amount of Ca^{2+} from the SR, called Ca^{2+}-induced Ca^{2+} release (CICR), via the SR Ca^{2+}

release channels, called the ryanodine receptor (RYR). This raises the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and binds to troponin C. Binding of calcium to troponin C, switches on the contractile machinery. Calcium must dissociate from troponin C in order for relaxation to occur, requiring that calcium must be transported out of the cytosol. Transport of calcium out of the cytosol is mediated by four pathways: SR Ca^{2+}-ATPase (SERCA) for re-uptake of Ca^{2+} by the SR, sarcolemmal Na^+/Ca^{2+} exchange, sarcolemmal Ca^{2+}-ATPase or mitochondrial Ca^{2+} uniport (Bers, 2002).
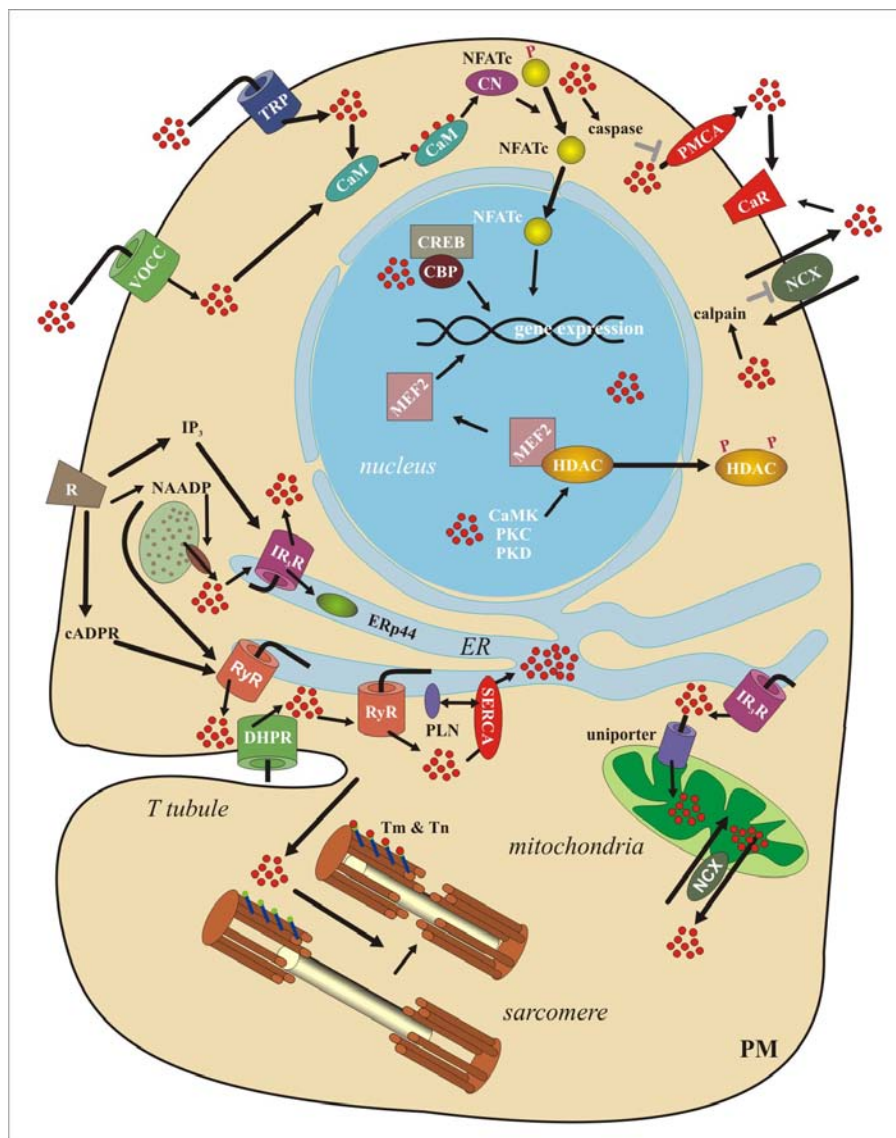
Figure 2.8. Components of Ca^{2+} signalling and organelles involved in Ca^{2+} homeostasis (Montell, 2005). Ca^{2+} ions being presented by red dots; cADPR, cyclic ADP-ribose; CaM,

calmodulin; CaMK, calmodulin dependent protein kinase; CaR, extracellular calcium-sensing receptor; CN, calcineurin; CREB, cAMP response element binding protein; DHPR, dihydropyridine receptor; HDAC, histone deacetylase; IP3, inositol triphosphate; IP3R, IP3 receptor; MEF2, myocyte transcription factor 2; NAADP, nicotinic acid adenine dinucleotide phosphate; NCX, Na^+/Ca^{2+} exchanger; NFAT, nuclear factor of activated T cells; PKC, protein kinase C; PKD, protein kinase D; PLN, phospholamban; PM, plasma membrane; PMCA, plasma membrane Ca^{2+} ATPase; RyR, ryanodine receptor; SERCA, sarcoplasmic and endoplasmic reticulum calcium ATPase; TM, tropomyosin; TN, troponin; TRP, transient receptor protein; VOCCs, voltage operated Ca^{2+} channels**.**

The expression of the calcium-sensing receptor (CaR), a G protein-coupled receptor, in cardiac tissue and cardiomyocytes was discovered by Wang and colleagues (2003). It is a key regulator for sensing calcium homeostasis, salt, water balance and osmotic regulation (Nearing *et al*., 2002). The main ligand for activation of CaR is Ca^{2+}, but it can also be activated by other cations (Handklogten *et al*., 2000). Physiological polyamine concentrations activate CaR and the efficacy is related to the number of positive charges, with spermine (4 positive charges) being more potent than spermidine (3 positive charges) in human embryonic kidney cells (HEK-293) (Quinn *et al*., 1997). Activation of CaR modulates a wide variety of proteins, including G proteins and phospholipase C, which in turn activates inositol 1,4,5-triphosphate production (IP_3). IP_3 subsequently increases intracellular Ca^{2+} release from the SR through the IP_3 receptor (Tfelt-Hansen, 2003). An increase in intracellular Ca^{2+} concentration in cardiomyocytes leads to increased cardiac activity but calcium overload also leads to apoptosis in cardiac I/R (Zhang & Xu, 2009).

Calmodulin is a Ca^{2+}-binding protein that regulates RYR opening by binding to it. The pump activity of SERCA is regulated by phospholamban (PLB) in an inhibitory manner. The SERCA2a isoform plays a central role in the heart for excitation-contraction coupling. Defects in the SERCA lead to altered contractile function (Periasamy & Huke, 2001).

S100 Ca^{2+}-binding protein A1 (S100A1), predominantly present in the heart, is associated with the sarcolemma, junctional and longitudinal SR, sarcomere, intercalated disc and mitochondria of ventricular cardiomyocytes (Schaub & Heizmann, 2008). Abnormal S100A1

gene expression leads to cardiomyopathy. At the molecular level, S100A1 interacts in a Ca^{2+}-dependent way with the ryanodine receptor (RYR2), SERCA2a, phospholamban, titin and the mitochondrial F1-ATP synthase (complex V). Stimulation of RYR2 by S100A1 increases CICR from the SR and by enforcing closure of the RYR2 channel, S100A1 reduces Ca^{2+} release from the SR. S100A1 binds to the N2B (or N2BA) isoforms and PEVK regions of titin in a Ca^{2+}-dependent manner. The N2B and PEVK elements bind to actin and this contributes to passive tension as it resists filament sliding during contraction. S100A1 is also involved in the mitochondrial energy production, by interacting with F1-ATP synthase. Increased concentrations of S100A1 increase the levels of ATP, and *vice versa* (Schaub & Heizmann, 2008).

2.4   The role of polyamines in mammalian cells

The polyamines (spermine, spermidine and putrescine), present in millimolar concentrations, play important roles in the cell. They are essential for normal cell growth, proliferation and differentiation, but can also cause neoplastic transformation and cell death (Janne *et al*., 1991). Polyamines are thus Janus-faced regulators that, depending on cell type and environmental signals, can promote growth or death. Abnormal expression of polyamines results in tumorigenesis, altered gene expression and induction of apoptosis (Cohen, 1998). Due to their cationic nature, polyamines interact with polyanions. Polyamines can interact with DNA and protect DNA from ROS (Pedreño *et al*., 2005). They also interact with RNA, nucleotide triphosphates, ion channels and other acidic substances (Igarashi & Kashiwagi, 2000).

Polyamine levels in cells are regulated by their biosynthesis, degradation, uptake and excretion (Igarashi & Kashiwagi, 2010). Putrescine is formed from ornithine by ornithine decarboxylase (Fig. 2.9). Decarboxylated S-adenosylmethionine is synthesized from S-adenosylmethionine by S-adenosylmethionine decarboxylase. These two enzymes are the rate-limiting enzymes in the synthesis of polyamines (Igarashi & Kashiwagi, 2010). Spermidine is synthesized from putrescine by spermidine synthase and spermine from spermidine by spermine synthase. In addition, spermidine is also formed from spermine by

spermine oxidase. Spermidine/spermine N-acetyltransferase and acetylpolyamine oxidase convert spermine to spermidine and spermidine to putrescine. A unique protein, antizyme, regulates the cellular polyamine content. It inhibits ornithine decarboxylase and aids in its degradation. Antizyme also inhibits polyamine uptake and enhances the excretion of polyamines (Mitchell *et al.*, 1994).

Polyamine oxidases regulate the levels of mono- and polyamines by oxidative deamination, to generate H_2O_2, aminoaldehydes and ammonia. Acrolein ($CH_2=CH-CHO$) is then spontaneously formed between aminoaldehyde (from spermidine) and aminodialdehyde (from spermine) (Yoshida *et al.*, 2009). Hydrogen peroxide induces cell death due to oxidative stress (Toninello *et al.,* 2004). Mitochondrial monoamine oxidase acts as a scavenger of other amines with different chemical structures, e.g. catecholamines and serotonin.

Although mammalian cells posses a tightly-regulated biosynthetic pathway for synthesis of polyamines, there exists an active polyamine uptake system in the extracellular medium, the polyamine transport system (Cullis *et al.*, 1999). This polyamine transporter was tested with a considerable range of polyamine analogues and they inhibit the uptake of spermidine (Cullis *et al.*, 1999). It seemed that the number of positive charges were a major determinant of binding to the polyamine receptor. This transport system can, in addition to natural polyamines, take up a wide range of substrates (Phanstiel *et al.*, 2000). Ghani and colleagues (2009) used polyamines as a vector to transport two toxic agents, 9-anthracenylmethyl-butanediamine and N1-anthracenylmethyl-4,4-triamine, to human leukemia cancer cells (HL-60). They observed a significant depletion of polyamines after treatment for 48 h and found that the toxicity of the two compounds increased when polyamine depletion occurred. Soulet and colleagues (2004) followed polyamine transport in CHO cells with a Spd-C_2-BODIPY probe (*N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl),*N'*-(*S*-[spermidine{N^4-ethyl}]thioacetyl)ethylenediamine). Their proposed model included import of polyamines by a plasma membrane carrier and sequestration into pre-existing polyamine-sequestering vesicles (PSV). These PSVs co-localized with acidic vesicles of the late endocytic compartment, which involves H^+ exchange through vacuolar-ATPase activity, and the *trans* Golgi network.
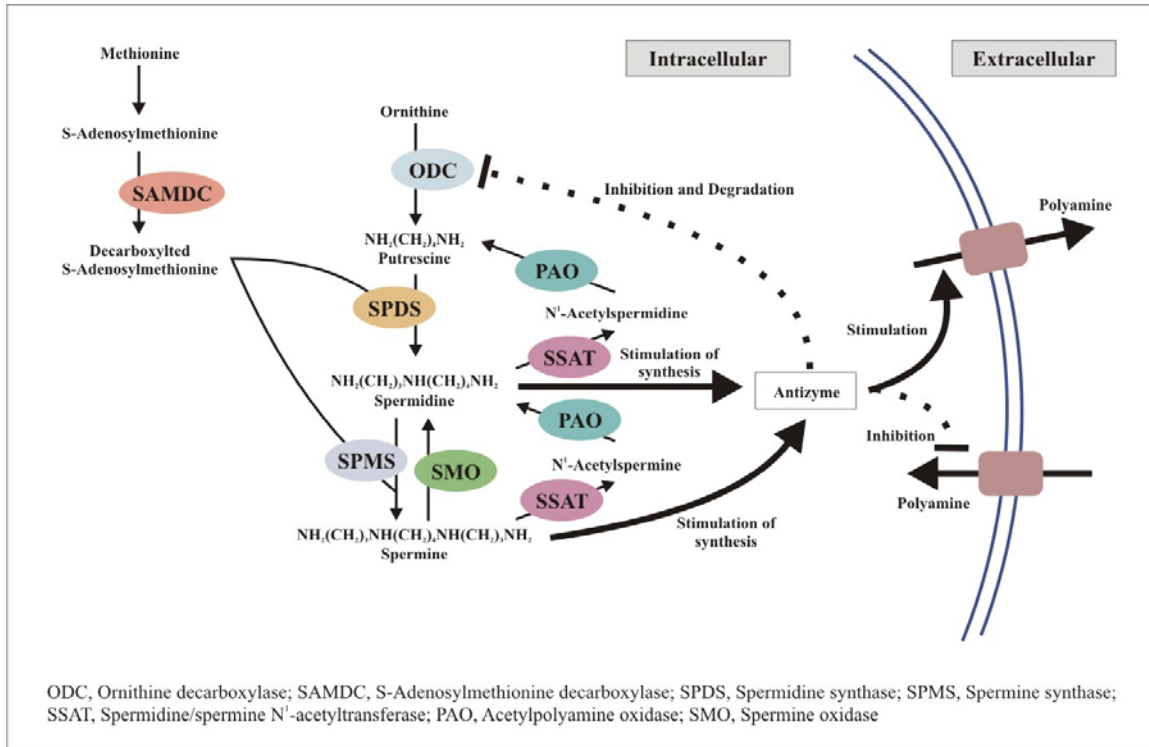
Figure 2.9 Synthesis and catabolism of the polyamines (Igarashi & Kashiwagi, 2009).

Polyamines induce muscle F-actin polymerization (Oriol-Audit, 1978). The chain length of polyamines determines the degree of F-actin polymerization, with maximum polymerization occurring with spermidine and spermine (Oriol-Audit, 1978). The dynamics of the cytoskeleton during cell proliferation and transformation were regulated by polyamines and ornithine decarboxylase, one of the rate-limiting enzyme of polyamine synthesis (Mäkitie *et al*., 2009). The activity of RHO A, a key modulator of actin cytoskeleton, was regulated by transglutaminase-catalyzed polyamination and the polyamination status of RHO A crucially influenced the progress of the cell cycle and rate of transformation in rat fibroblasts infected with viral sarcoma (v-src), a proto-oncogenic tyrosine kinase (Mäkitie *et al*., 2009).

The exact role of the polyamines in the heart has not yet been elucidated. It is now known that cardiac hypertrophy and simulated ischemia are characterized by an increase in

polyamine levels and are coupled with excessive apoptosis (Diez *et al.*, 1998; Tantini *et al.*, 2006). Spermine causes a dose-dependant decrease in ventricular contraction which follows the release of ATP in the effluent during heart perfusion studies (Guevara-Balcázar *et al.*, 2003). Polyamines, especially spermine, interact with myofibrillar proteins to reduce Ca^{2+} binding affinity (Harris *et al.*, 2000). Polyamines bind weakly and reversibly to the mitochondrial membrane at specific sites. This allows for their transport into the matrix and interaction with Ca^{2+} transporters. In addition, spermine plays an important role in Ca^{2+} homeostasis (Salvi & Toninello, 2004). Spermine increased intracellular Ca^{2+} in rat cardiomyocytes from adult hearts due to activation of the calcium-sensing receptor (CaR) (Wang *et al*., 2003). Furthermore, polyamines present in cardiac muscle act as permeable cationic channel blockers of the ryanodine receptors (Uehara *et al*., 1996).

Spermine is an inhibitor of MPTP in cardiomyocyte mitochondria (Lapidus & Sokolove, 1992). Spermine, at 10-100 µM, induces the release of cytochrome *c* from isolated rat heart mitochondria and is more potent than spermidine in this respect (Stefanelli *et al.*, 2000). The release of cytochrome *c* is not blocked by CsA, an inhibitor of the MPTP. Thus, the release of cytochrome *c* is not a consequence of opening of the MPTP. Maccarrone *et al*. (2001) reported that the release of cytochrome *c* from the mitochondria by spermine is paralleled by depolarization of $\Delta\Psi_m$, and thus disruption of the mitochondrial membrane integrity. They further reported that with the addition of pargyline, an inhibitor of amine oxidases, or catalase, an enzyme that converts H_2O_2 to water and oxygen, they abolished the release of cytochrome *c* and dissipation of the $\Delta\Psi_m$. They speculated that it was the polyamine oxidation products, rather than the polyamines themselves, that disrupted mitochondrial integrity.
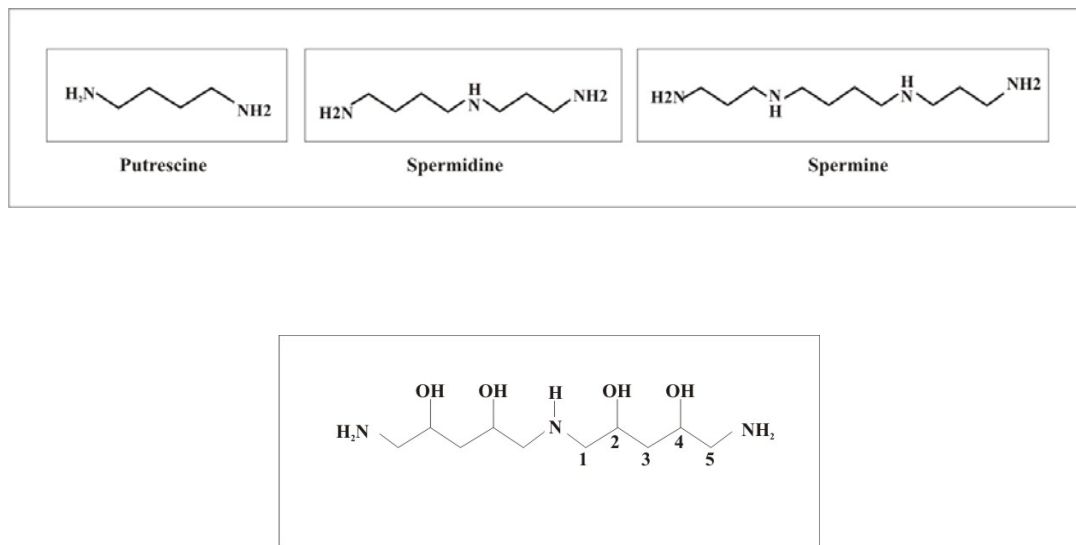
Figure 2.10 Structure of the natural polyamines and pavetamine (Bode *et al*., 2010).

2.5   Death of cardiomyocytes: apoptosis, autophagy and necrosis

Three types of programmed cell death are described in the literature: apoptosis, autophagy and necrosis (Table 2.1). Many studies reported on apoptotic cell death but necrosis was regarded as accidental cell death. Only recently, it became known that necrosis is also an organized cell death pathway. All three death pathways are interlinked and the final outcome may depend on the type of insult, its duration, as well as intracellular metabolic capacity (Loos & Engelbrecht, 2009). Complex interactions exist between autophagy and apoptosis (Levine & Yuan, 2005). Beclin-1 (BECN1), participates in autophagosome formation, but it also interacts with the anti-apoptotic protein, Bcl-2 and thus prevents autophagy (Pattingre *et al*., 2005). The death decision of cells is a complex process, as "cross-talk" between different cell death pathways exists (Lockshin & Zakeri, 2004). Therefore, it seems that autophagy promotes cell survival by avoiding a metabolic crisis and delays the onset of apoptosis and necrosis (Loos & Engelbrecht, 2009). However, it has been reported that intracellular ATP concentrations determine the eventual type of cell death (Leist *et al*., 1997).

2.5.1   Apoptosis

Apoptosis (programmed cell death I) is an active gene-directed process (Ohno *et al*., 2008). There exist two pathways for apoptosis: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Skommer *et al*., 2007). Mitochondria are closely involved in the process of apoptosis. Several stress signals affect the mitochondria, namely reactive oxygen species (ROS), nitric oxide (NO), altered redox state, as well as increases in Ca^{2+} concentrations (Green & Leeuwenburgh, 2002). The MPTP opens during apoptosis, permitting the influx and efflux of molecules with a molecular weight of less than 15 kDa (Zoratti & Szabo, 1995) and consequentlythe $\Delta\Psi_m$ depolarizes (Kroemer & Reed, 2000).

Apoptotic cell death is characterized by cell shrinkage, pre-lytic DNA fragmentation (ladder pattern on gel electrophoresis), chromatin condensation (pyknosis) and chromatin fragmentation (karyorrhexis), exposed phosphatidyl serine (PS), cytochrome *c* release from the mitochondria into the cytoplasm and activation of the caspase family of proteases (cytosolic aspartate residue-specific cysteine proteases) (Kunapuli *et al.*, 2006). Apoptosis is characterized by an intact plasma membrane and requires ATP (Leist, *et al*., 1997). Caspase-activated DNAase induces DNA fragmentation of 200 base pairs (Van Wijk & Hageman, 2005). Caspase-independent apoptosis can proceed via translocation of an apoptosis-inducing factor (AIF), a mitochondrial intermembrane protein, to DNA and can induce large-scale DNA fragments of 50 base pairs (Kim *et al.*, 2006). AIF inhibits protein synthesis by interaction with the eukaryotic translation initiation factor 3 subunit p44 (dIF3g) (Kim *et al*., 2006). The end stage of heart failure (due to coronary artery disease, hypertension, valvular heart disease, myocarditis and diabetes) is death of the myocytes (Regula *et al*., 2003). Apoptosis was demonstrated in many experimental models of heart failure such as ischemia, ischemia-reperfusion (I/R), hypoxia, Ca^{2+} excess, oxidative stress, rapid pacing, gene induction, sustained stretching and doxorubicin use (Kunapuli *et al.*, 2006).

2.5.2   Autophagy

Long-lived proteins, macromolecules, membranes and whole organelles are degraded by autophagy via the lysosomes, thereby controlling the rate of turnover (Levine & Klionsky, 2004). Autophagy is activated during stress conditions such as amino acid starvation, unfolded protein response or viral infection, as part of the cell's survival. Autophagy is responsible for organelle turnover and occurs in four distinct steps: induction, formation of autophagosome, autophagosome docking and fusion with the lysosome or vacuole, as well as autophagic body breakdown (Kunapuli *et al*., 2006).   Knaapen and co-workers (2001) demonstrated that cardiomyocytes preferentially undergo caspase-independent autophagic cell death during heart failure. However, as discussed above under the modes of cell death, various factors determine the eventual cell death fate and the availability of ATP which is a key determinant. Autophagy is a well regulated process, where under normal circumstances, growth factors activate class I phosphatidylinositol-3 kinase (PIK3) proteins. In turn, these then activate the mammalian target of rapamycin (mTOR) through the serine/threonine protein kinase (PKB/AKT) pathway (Abeliovich, 2004). Active mTOR inhibits an autophagy-related protein, ATG1, a key regulator of autophagy induction. During starvation, mTOR is not activated and ATG1 is able to form an ATG1 protein-kinase autophagy-regulatory complex that induces autophagy (Abeliovich, 2004). Small decreases in ATP lead to activation of AMP-activated protein kinase, which inhibits mTOR and protein synthesis (Meijer & Dubbelhuis, 2004). 3-Methyladenine is a specific inhibitor of autophagy, through inhibition of PIK3 (Seglen & Gordon, 1982).

The golden standard for assessing autophagy, is through the use of electron microscopy. This method shows the typical features of autophagy which include swollen SR and mitochondria, double-membraned autophagosomes/vacuoles, with the absence of chromatin condensation (Herrera *et al*., 2006). The microtubule-associated protein 1 light chain 3 (LC3) is a biomarker for autophagy, as it forms part of a structural component during autophagosome formation (Martinet *et al*., 2007). LC3 is lipidated during autophagosome formation and this LC3-phospholipid conjugated (LC3-II) is localized on autophagosomes. Beclin-1 has also been used to detect autophagy (Yan *et al*., 2005). The typical characteristics of autophagy have been reported in heart failure and the incidence of autophagy in heart failure has been

found to be greater than the incidence of apoptosis (Martinet *et al*., 2007). Autophagy has been described in heart failure caused by dilated cardiomyophathy (Kostin *et al*., 2003), valvular and hypertensive heart disease (Hein *et al*., 2003), chronic ischemia (Yan *et al*., 2005) and in human hibernating myocardium (Elsässer *et al*., 2004). A hibernating myocardium is described as a state of persistently impaired myocardial contractile function at rest due to reduced coronary blood flow (Rahimtoola, 1989).

2.5.3   Necrosis

Initially it was thought that necrosis is accidental cell death, but lately it has been shown that necrosis is an organized cell death pathway (Festjens *et al*., 2006). If the classic apoptotic cell death fails, other caspase-independent cell death pathways can occur, such as necrosis or autophagy (Festjens *et al*., 2006). Necrosis is characterized by cytoplasmic swelling, dilatation of cytoplasmic organelles-especially the mitochondria, irreversible plasma membrane damage and post-lytic random DNA digestion (smear pattern on gel electrophoresis) (Grooten *et al*., 1993). Necrosis is not accompanied by the typical apoptotic features such as internucleosomal DNA cleavage and nuclear condensation or by features of autophagy (Hitomi *et al*., 2008). Goldstein and Kroemer (2006) described the sequence of intracellular events for necrosis as follows: early signs of mitochondrial dysfunction, namely production of ROS and mitochondrial swelling, ATP depletion, Ca^{2+} overload, perinuclear clustering of organelles, activation of proteases (in particular calpains and cathepsins), lysosomal rupture and ultimately plasma membrane rupture. Necrotic cell death can be induced by ligands that bind to plasma membrane receptors, like tumor necrosis factor α (TNF α), which is an inflammatory cytokine (Goossens *et al*., 1995). Apoptosis can be triggered by partial selective lysosomal permeabilization, but a massive breakdown of lysosomes will result in unregulated necrosis (Bursch, 2001). Necrosis is characterized by ATP depletion, ion disregulation, mitochondrial and cellular swelling (Marx *et al*., 2006), as well as activation of cysteine proteases, Ca^{2+}-activated calpain, cathepsin and caspases (Yamashima, 2000).

Table 2.1 Comparison of typical features of cell death by the three programme cell death pathways

Type of cell death	Morphology of cells	Reference
Apoptosis	Cell shrinkage Pre-lytic DNA fragmentation (ladder pattern on gel electrophoresis) Chromatin condensation (pyknosis) and chromatin fragmentation (karyorrhexis) Exposed phosphatidyl serine (PS) Cytochrome *c* release from the mitochondria into the cytoplasm Activation of the caspase family of proteases (cytosolic aspartate residue-specific cysteine proteases) Requires ATP	Kunapuli *et al.*, 2006
Autophagy	Swollen SR and mitochondria Double-membraned autophagosomes/vacuoles No chromatin condensation Presence of microtubule-associated protein 1 light chain 3 (LC3) Beclin-1 expression Generates ATP	Yan *et al.*, 2005; Martinet *et al.*, 2007, Loos & Engelbrecht, 2009.
Necrosis	Cytoplasmic swelling Dilatation of cytoplasmic organelles, especially the mitochondria Irreversible plasma membrane damage Post-lytic random DNA digestion (smear pattern on gel electrophoresis) LDH leakage ATP depletion	Grooten *et al.*, 1993

2.6 Cardiac hypertrophy

During cardiac hypertrophy, the immediate early gene family (c-JUN, c-FOS and early growth response gene 1) is activated and is then followed by reactivation of the expression of certain foetal genes like β-myosin heavy chain (β-MHC) and the natriuretic peptides *viz.* atrial natriuretic peptide, brain natriuretic peptide and C-type natriuretic peptide (Purcell *et al*., 2001; Gardner, 2003). The C-terminal peptides bind to the natriuretic peptide receptors (guanylyl cyclase receptors). This binding converts guanosine triphosphate (GTP) to the second messenger 3´,5´-cyclic guanosine monophosphate, which activates intracellular protein kinases and inhibits hypertrophy (Gardner *et al*., 2007). An increase in the expression of the β-MHC isoform decreases the ATPase activity, which then lowers the contraction rate, which is an important adaptation to altered workload (Lowes *et al*., 1997).

2.7 Important signalling pathways in the heart

2.7.1 Mammalian target of rapamycin (mTOR)/phosphoinositide 3-kinase/Akt signalling

The key regulator of protein synthesis for cell growth, is the mammalian target of rapamycin mTOR (Wang & Proud, 2006). mTOR stimulates protein synthesis by activating p70 ribosomal S6 kinase and by inhibiting eukaryotic translation initiating factor 4E-binding protein 1, which is a repressor of translation initiation (Sarbassov *et al*., 2005). mTOR senses the availability of amino acids and ATP levels (Chen & Fang, 2002). Rapamycin is an anti-tumor drug that prevents protein synthesis and arrests the cell cycle in the G1 phase (Asnaghi *et al*., 2004). Rapamycin is also a strong inducer of autophagy (De Meyer & Martinet, 2009). mTOR belongs to the phosphoinositide 3-kinase (PI3K) pathway and is activated by tyrosine kinase growth factor receptors such as epidermal growth factor receptor (EGFR) and insulin-like growth factor-1 receptor (IGF-1R), cell adhesion molecules such as integrins, G-protein-coupled receptors (GPCRs), and oncogenes such as Ras. (Vogt, 2001; LoPiccolo *et al*., 2008) (Fig. 2.11 for this pathway). An upstream regulator of mTOR is the serine/threonine protein kinase B (PKB)/AKT (Nave *et al*., 1999). mTOR controls the transcription activator, signal transducer and activator of transcription 3 (Yokogami, *et al*., 2000). Phosphoinositide 3-

kinase (PIK3) is involved in cell growth, proliferation, survival, migration, metabolism and other biological responses (Foukas & Okkenhaug, 2003). PIK3 exerts cardioprotective effects in the heart through activation of key proteins, including Akt. Activated Akt prevents apoptosis induced by different kind of insults (Fujio *et al*, 2000). Three isoforms of Akt exist, namely Akt 1 (PKBα), Akt 2 (PKBβ) and Akt 3 (PKBγ) (Matsui & Rosenzweig, 2005). Regulation of Akt is accomplished by protein phosphatase 2A. Akt phosphorylates the Forkhead transcription factor, leading to reduced transcription of pro-apoptotic molecules (Brunet *et al*., 1999).

2.7.2   Nuclear factor kappa beta (NF-κB)

The redox-sensitive inducible transcription factor NF-κB plays a central role in immune responses, inflammation, cell survival, differentiation and proliferation (Xiao, 2004). Nuclear factor kappa beta is retained in an inactive form in the cytoplasm by the inhibitor of NF-κB (IκB). The activation of the IκB kinase results in IκB phosphorylation, triggering its ubiquitination and proteasomal degradation. Free NF-κB translocates to the nucleus where it binds to target sequences. This promotes or inhibits transcription through co-activator or co-repressor recruitment (Hayden & Ghosh, 2008).

2.7.3   MAPK signalling

Other pathways that are involved in cardiac hypertrophy (CH) are the mitogen-activated protein kinase (MAPKs) and AKT pathways (Hayata *et al*., 2008). The MAPKs are a family
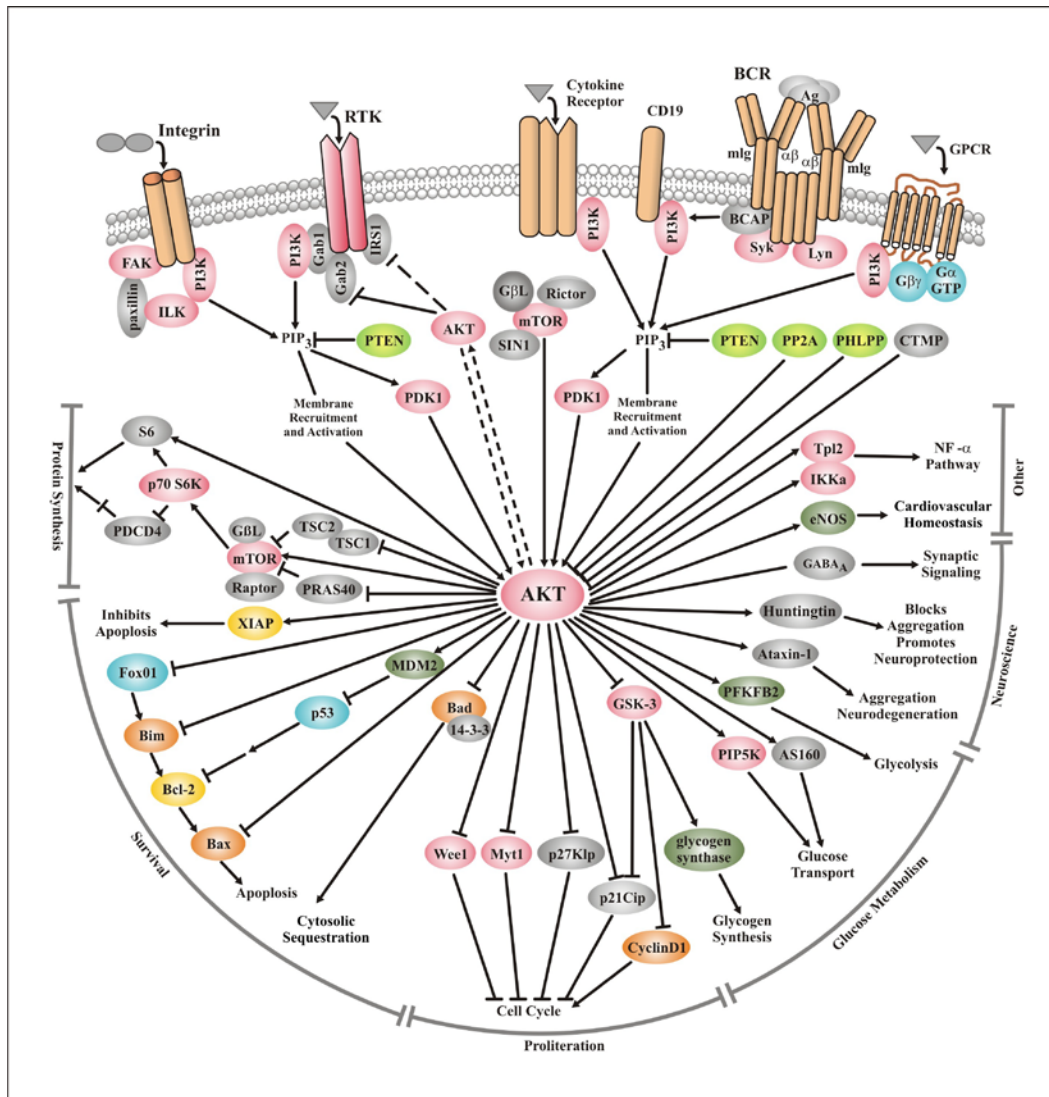
Figure 2.11 Schematic diagram of PI3K/Akt/mTOR signalling pathway (http://www.cellsignal.com/pathways/akt-signaling.jsp).

AS160, a substrate of Akt, Bax, Bcl-2-associated X protein, BCAP, B cell adaptor for PI3K, Bcl-2, B-cell lymphoma 2, BCR, B cell receptor, Bim, proapoptotic protein of the Bcl-2 family, CTMP, carboxyl-terminal modulator protein, eNOS, endothelial nitric oxide synthase, FAK, focal adhesion kinase, Foxo1, transcription factor, GABA$_A$, gamma aminobutyric acid, GSK-3, glycogen synthase kinase 3, IKKα, IκB kinase alpha, ILK, integrin-linked kinase, IRS1, Insulin receptor 1, Jak1, Janus kinase, Lyn, Src-related tyrosine kinase, MDM2, ubiquitin ligase, Myt1, myelin transcription factor, p53, p53 tumor suppressor protein, PDCD4, programmed cell death 4, PDK1, pyruvate dehydrogenase kinase 1, PFKFB$_2$, 6-

phosphofructo-2-kinase/fructose-2,6-biphosphatase 2, PHLPP, PH domain and leucine rich repeat protein phosphatase, PIP_3, phosphatidylinositol 3,4,5-trisphosphate, PIP5K, phosphatidylinositol 4-phosphate 5-kinase, PP2A, protein phosphatase 2, PRAS40, proline-rich Akt/PKB substrate 40 kDa, PTEN, phosphatase and tensin homolog, Raptor, regulatory associated protein of mTOR, Rictor, rapamycin insensitive companion of, mTOR, RTK, receptor tyrosine kinase, SIN1, SAPK-interacting protein 1, Syk, spleen tyrosine kinase, Tpl2, oncoprotein kinase, TSC1/2, tuberous sclerosis complex, Wee1, protein kinase, XIAP, X-linked inhibitor of apoptosis

of serin-threonine kinases that are activated via a variety of stimuli. Extracellular signal-related protein kinase, p38-MAPK and c-JUN NH_2-terminal protein kinase (JNK) are three major MAPKs, activated during ischemia and reperfusion (I/R) in the heart (Bogoyevitch *et al*., 1996; Knight & Buxton, 1996; Pearson *et al*., 2001). Activated MAPKs interact with protein kinases (i.e. mitogen- and stress-activated protein kinase, MSK1), cytoskeletal proteins , transcription factors and αB-crystallin (Aggeli, *et al*., 2008). The p38-MAPK in the heart is involved in cardiac gene expression, inflammation, energy metabolism, contractility, proliferation and apoptosis (Baines & Molkentin, 2005; Engel, 2005; Clerk & Sugden, 2006). Activated p38-MAPK has an influence on a number of transctiption factors, including myocyte enhancer factor 2, activating transcription factors (ATF-2 and ATF-6), NF-κB and E-26-like protein 1 (Kerkelä, 2003). Myocyte enhancer factor 2 is a transcription factor that is expressed in cardiac muscle and is required for cardiogenesis (Ren *et al*., 2007). JNKs and p38 kinases are called the stress-activated protein kinases because they function as transducers of stress or injury responses (Liang & Molkentin, 2003). p38-MAPK is involved in ischemic injury (Liu *et al*., 2005). Activators of p38-MAPK include MAPK kinase 3 (MKK3) and MAPK kinase 6 (MKK6), which are activated by phosphorylation on Ser/Thr residues by MAPK kinase kinase (MAPKKKs). These MAPKKKs are partly activated in response to oxidative stress, heat shock, UV irradiation, hypoxia, ischemia, and pro-inflammatory cytokines, interleukin 1 and tumor necrosis factor (TNF). (Rainaud *et al*., 1996). MAPK-activated protein kinase 2 phosphorylates heat shock protein 27, lymphocyte-specific protein 1 and cAMP response element-binding protein (Bassi *et al*., 2008). Activation of the p38-MAPK during myocardial ishaemia can be lethal, but under other

circumstances, activation of p38-MAPK can protect the heart (Bassi *et al*., 2008). Dual-specific MAPK phosphatases (MKPs) deactivate p38-MAPK (Keyse, 1999).

2.7.4   G protein-coupled receptors (GPCRs)

The GPCRs are dedicated to cell-cell communication. They regulate second messengers and ion channel activity. GPCRs are activated by their ligands, which lead to a conformational change. This change causes the GPCR to interact with heterotrimeric G proteins (Bockaert *et al*., 2004). A GDP to GTP transition occurs within the G protein and the Gα-GTP and Gβγ subunits. GPCRs also interact with GPCR interacting proteins (Bockaert *et al*., 2004). GPCRs are crucial in cardiovascular function and the adrenergic receptors (ARs) are GPCRs that are involved in the hypertrophic response (Barry *et al*., 2008). α-Adrenergic receptors activate phospholipase C (PLC) that hydrolyses α-Adrenergic receptors activate phospholipase C (PLC) that hydrolyses phosphoinositol 4,5-biphosphate to inositol 1,4,5-triphosphate and diacylglycerol (DAG) (Arimoto *et al*., 2006). DAG activates protein kinase C (PKC), which is part of the development of concentric hypertrophy (Takeishi *et al*., 2000). β-Adrenergic receptors that are coupled to the $G_{\alpha s}$ subunit of the heterodimeric G protein, activate adenyl cyclase, causing accumulation of cAMP and consequently activation of protein kinase A (PKA). This results in the phosphorylation of various proteins involved in cardiac contraction: L-type calcium channels (I_{Ca-L}), ryanodine receptors (RYRs), phospholambin (PLB) and troponin (TN) (Marian, 2006). It is interesting to note that polyamines stimulate G proteins in peritoneal mast cells through phospholipase C (PLC) (Bueb *et al*., 1992).

2.8   Protein quality control (PQC)

Cellular protein synthesis occurs in cytosolic free ribosomes, but depending on cell type can occur in the rough endoplasmic reticulum (ER). Membrane proteins and proteins for secretion are synthesized in the ER, where proper folding must take place (Blobel, 2000). The ER-associated protein quality control supports protein refolding, prevents unfolded proteins from aggregating and selectively removes misfolded polypeptides (Wang *et al*., 2008). In cardiomyocytes, myofibrillar proteins occupy more than 80 % of the cell volume and the PQC of these cells is not a function of the ER. In order for proper folding to take place, chaperones

are synthesized to prevent proteins from misfolding (Willis *et al*., 2009). Targeted proteolysis of misfolded proteins is accomplished by the ubiquitin-proteasome system (UPS) (Wang & Robbins, 2006).

Chaperones assist unfolded polypeptides to fold correctly. For each subcellular compartment (e.g. mitochondria, the ER, the nucleus, and the cytosol) there is a different set of chaperones. Chaperones serve as sensors for misfolded polypetides, bind them and prevent aggregation. In stressed cardiomyocytes, there is an increase in the synthesis of chaperones to handle increased protein misfolding. The heat shock proteins (HSP) are induced in cardiomyocytes during stress. Some of these HSP chaperones are capable of refolding the proteins that have been denatured under stress, while others escort terminally misfolded proteins for degradation. The most studied chaperones in the heart are HSP90, HSP70, carboxyl terminus of HSP70-interacting protein, HSP20 and αβ-crystallin. In a number of cardiac pathologies, the PQC is inadequate, resulting in congestive cardiac failure. The formation of protein aggregates can impair the UPS and activate autophagy (Rubinsztein, 2006). Although autophagy is a death signalling pathway, it also plays a crucial role in PQC, especially in pathological conditions. Autophagy is capable of degrading excessive or defective organelles and there is a link between autophagy and ER-associated degradation (ERAD).

2.9 The unfolded protein response (UPR)

The protein folding machinery, including numerous chaperones, proteins and factors, ensures efficient nascent protein folding. Any disturbance of this folding machinery will lead to accumulation of misfolded proteins, which will trigger events to augment folding capacity. The UPR is activated by stresses that impair the protein folding in the rough ER (McMillan *et al*., 1994). Optimal protein folding depends on the following factors: the correct redox state in the ER, suitable levels of glycosylation substrates and glycosylation enzymes, SR Ca^{2+} and chaperones (Glembotski, 2008). When ER protein folding is impaired, the accumulation of misfolded, dysfunctional proteins signals the initiation of ER stress (Chang *et al*., 1987). The proximal ER transmembrane effectors of the UPR are: protein kinase R-like ER kinase (PERK) (Shi *et al*., 1998), activating transcription factor-6 (ATF6) (Zhu *et al*., 1997) and

inositol-requiring enzyme-1 (IRE-1) (Mori *et al*., 1993). With efficient ER protein folding, the ER luminal domains of these three effectors are bound to the ER-resident chaperone, glucose-regulated protein 78, thereby keeping these effectors inactive (Lee, 2001). However, when misfolded proteins begin to accumulate, glucose-regulated protein 78 translocates to the misfolded proteins to aid in folding. Activated PERK phosphorylates eukaryotic initiation factor 2α, leading to decreased translation of most cellular mRNAs (Bertolotti *et al*., 2000). Upon ER-stress, IRE-1 exhibits a novel endoribonuclease activity, which cleaves the mRNA of active X-box binding protein-1. This splicing event generates a new transcript that encodes for an active form of X-box binding protein-11, a transcription factor that induces numerous ER stress response genes (Calfon *et al*., 2002). ATF6 translocates to the Golgi apparatus, upon ER stress. Here ATF6 is cleaved by two proteases, and the cytosolic region of ATF6 translocates to the nucleus, leading to the transcriptional regulation of ER stress response genes (Ye *et al*., 2000). The genes that are induced upon ER stress, encode proteins that improve the folding of nascent proteins in the ER lumen and enable the degradation of misfolded proteins. This degradation is performed by the ER-associated protein degradation (ERAD). ERAD causes retrotranslocation of the unfolded polypeptide into the cytosol followed by ubiquitination and proteasomal degradation, or targeting parts of the ER to lysosomes through autophagy (Kincaid & Cooper, 2007).

2.10   The ubiquitin-proteasome system (UPS)

The UPS system is a non-lysosomal, ATP-requiring system responsible for the degradation of ubiquitinated proteins that is recognized by the 26S proteasome (Glickman & Ciechanover, 2002). The barrel-shaped 20S proteasome forms the proteolytic core of the 26S proteasome. Three major peptidase activities have been assigned to the 20S proteasome: chymotrypsin-like, trypsin-like and caspase-like activities (Wang *et al*., 2006; Willis & Patterson, 2006). Damaged and misfolded proteins are degraded by the UPS, as well as intracellular proteins (Hochstrasser, 1995). The C-terminus of ubiquitin is covalently attached to the ε-amino group of specific lysine residues in the substrate protein. This ubiquitination of target proteins involves three enzyme families: E1, an activating enzyme; E2, a conjugating enzyme that carries the ubiquitin; and E3, a ligase that recognizes the target protein and transfer of

ubiquitin from E2 (Balasubramanian *et al*., 2006). The extent of ubiquitination determines the fate of target proteins. The 26S proteasome recognizes substrates with at least 4 poly-ubiquitin chains linked to their lysine 48 which is a signal for degradation (Willis & Patterson, 2006). Mono-ubiquitination of lysine residues or ubiquitination at Lys63, may signal for a non-proteolytic fate for modified proteins. This can result in the internalization and sorting of ion channels, receptors and junctional complexes to the endocytic environment (Bonifacino & Traub, 2003). Histone modification, transcription and DNA repair are modulated by mono-ubiquitination (Hicke, 2001). In the heart, the UPS regulates cardiac membrane channels and receptors, β2-adrenergic signalling, signal transduction and transcription factors (Willis & Patterson, 2006). In cardiac I/R injury, the proteasome is inhibited with accumulation of ubiquitinated proteins (Powell *et al*., 2005). Activation of caspases inhibits proteasome function and leads to apoptosis (Sun *et al*., 2004). The UPS is activated during cardiac hypertrophy (Depre *et al*., 2006). Muscle-specific ring finger proteins (MURFs) MURF-1, MURF-2 and MURF-3 are a subfamily of E3 ubiquitin ligases, expressed in cardiac and skeletal muscle (Spencer *et al*., 2000). MURF-1 is a microtubule-associated protein (Spencer *et al*., 2000) and interacts with titin at the M-band of the sarcomere (McElhinny *et al*., 2002). MURF-1 and MURF-2 interact with titin, nebulin, TNI and TNT, myotilin and T-cap (Witt *et al*., 2005). MURF-3 interacts with four and a half LIM domain protein (FHL2) and γ–filamin, and therefore controls their degradation (Fielitz *et al*., 2007a). Fielitz *et al.* (2007b) demonstrated that MURF-1 and MURF-3 interact with β/slow MHC and MHCIIa and play a central role in the maintenance of skeletal and cardiac structure and function. A proteomic study was undertaken to identify the ubiquitinated proteins in the mouse heart by way of a transgenic mouse model expressing a plasmid with an ubiquitin tag (Jeon *et al*., 2007). The cytosolic proteins identified in this manner included components of the contractile fibres, namely MHC α and β, TPM, titin, MYBPC, desmin and actinin 2 and 4. These findings underline the importance of the UPS in the turnover of the cardiac contractile and cytoskeletal proteins.

During myocardial ischemia, oxidation of proteins occurs, which can be measured by an increase in protein carbonyls and mixed disulfides after perfusion of isolated heart (Park *et al*., 1991). The proteasome plays a significant role in the removal of oxidized proteins during myocardial ischemia in an ubiquitin-independent manner (Divald & Powell, 2006). A

transgenic mouse model was also used to investigate the role of the UPS in the cardiotoxicity of doxorubicin therapy (Kumarapeli *et al*., 2005). These authors reported that doxorubicin enhanced UPS function in the heart and cultured cardiomyocytes.

2.11   Other proteases in cardiomyocytes: calpains, cathepsins and caspases

Lysosomal proteases, such as cathepsin D, are activated during the initial phase of ischemia and require an acidic pH for activity (Wildenthal, *et al*., 1978). A number of myofilament proteins, namely actin, myosin, TPM and troponin, are reported to be degraded in ischaemic human left ventricles (Hein *et al*., 1995). Protein degradation and loss were studied in rat hearts subjected to ischemia and ischemia/reperfusion (I/R) (Van Eyk *et al*., 1997). During ischemia, there was an increased loss and degradation of α-actinin and troponin I. During I/R, degradation of MLC1 was also found. These authors concluded that the changes involved in myocardial function associated with I/R is an altered response of the myofilaments to Ca^{2+}. The degradation and loss of some of these proteins was attributed to Ca^{2+}-dependent proteases, which were activated during the Ca^{2+} overload after I/R (Gross *et al*., 1999). Calpain, which is located near the Z-line, is one of the Ca^{2+}-dependent proteases that play a role in ischemia (Goa *et al*., 1997). In an immunohistochemical study using human hearts with dilated cardiomyopathy, the intensity of titin fluorescence was reduced, frequently disorganized or almost completely absent (Hein *et al*., 1994). These authors concluded that the loss of titin, myosin and the thin filament complex correlated with the reduction in cardiac function. Multimeric complexes of sarcomeric proteins cannot be degraded by the proteasome (Solomon & Goldberg, 1996). Calpain-1 is needed to dissociate sarcomeric proteins from the myofibril before the UPS is able to degrade those (Galvez *et al*., 2007). The SR plays a central role in cardiac contractility due to its ability to regulate intracellular Ca^{2+} (Bers, 2002). In an I/R rat heart model, it was demonstrated that the SR function and gene expression was altered (Temsah *et al*., 1999). SR Ca^{2+}-cycling and SR regulatory proteins were shown to be a target for calpain action (Singh *et al*., 2004). Upon leupeptin treatment, an inhibitor of calpain, there was a recovery of the major SR Ca^{2+}-handling proteins, RYR and SERCA2a, and its regulator phospholambin.

Myofibrillar proteins are degraded by the lysosomal proteases. TNT is being degraded by cathepsin H, while cathepsin B hydrolases MHC, TNT, TNI and TPM (Bechet *et al*., 2005). Most of the myofibrillar proteins are degraded by cathepsin L, except TNC and TPM (Matsukura *et al*., 1981).

2.12   Lysosomotropism

Lysosomes are vesicles that contain high concentrations of acid hydrolases which are active in an acidic pH of 4-5 (Kirschke & Barrett, 1987). The vacuolar (V)-type ATPase proton pump maintains the acidic pH of the lysosome. The accumulation of basic/cationic compounds inside acidic organelles, like the late endosome or lysosome, are termed lysosomotropism (De Duve *et al*., 1974). Several cell types form multiple and large vacuoles when treated with concentrated amine drugs, like procaine, procainamide, nicotine and atropine (Morisette *et al*., 2008). Bafilomycin A1, a V-ATPase inhibitor, completely prevented vacuole formation by diverse amine drugs (Morisette *et al*., 2004). Monoamines and diamines, because of their weak basic lipophilic character, are commonly employed to study vacuolar acidification (Millot *et al*., 1997). In their neutral form, these compounds are membrane-permeant, but once protonated, they would accumulate in acidic vesicles and become membrane-impermeant. Leakage of lysosomal enzymes can cause apoptosis or necrosis (Wang *et al*., 2006). Oxidative stress, accumulation of redox-active iron and lipid peroxidation cause lysosomal rupture (Parent *et al*., 2009). Pronounced lysosomal leakage and rupture result in necrosis, while moderate lysosomal leakage induces apoptosis (Bursch, 2001).

2.13   Justification for this study and hypothesis

The ultimate aim of this gousiekte research project is to develop preventative or treatment options for gousiekte. In order to achieve this, the mechanism of toxicity of pavetamine must first be clarified. Polyamines, present in millimolar quantities, play essential roles in cells, but the function of polyamines in cardiac cells is still largely unknown. Polyamines can promote cell growth or cell death, they can influence gene expression and are involved in Ca^{2+} homeostasis. During the catabolism of polyamines by polyamine oxidases, H_2O_2 is generated

and can cause extensive damage to the cell. The generation of ROS cause protein oxidation, thus forming protein aggregates, which are degraded by the UPS. Multimeric complexes of sarcomeric proteins must first be degraded by calpain 1 before the UPS can further digest them. Pavetamine can possibly interfere with the metabolism of polyamines, their biosynthesis and with their transport. Polyamines change the Ca^{2+} affinity of contractile proteins. Pavetamine can behave like other amine-containing compounds, such as NH_4Cl, chloroquine and methylamine, where it gets trapped inside acidic vesicles, causing multiple and large vacuoles, so called lysosomotropism.

In summary, the following cellular effects have been reported for pavetamine:

- It damages the mitochondria in the heart of rats and sheep and ATP levels are reduced (Snyman *et al*., 1982; Prozesky *et al*., 2005).

- It damages the SR in the hearts of sheep and rats and causes reduced uptake of calcium by the sarcoplasmic reticulum (Pretorius *et al*., 1973b; Prozesky *et al*., 2005).

- Ultrastructurally, myofibrillar loss and degeneration are typically observed in hearts of sheep and rats exposed to pavetamine (Schutte *et al*., 1984; Kellerman *et al*., 2005; Prozesky *et al*., 2005; Prozesky, 2008).

- Pavetamine also causes inhibition of protein synthesis in rat hearts (Schultz *et al*., 2001).

The working heart has a high rate of energy utilization to enable rhythmic contraction. Impaired ATP production will hamper contractility. Damaged mitochondria and SR are degraded in the lysosomes by autophagy. This is recognized as a cell survival mechanism. Autophagy can also be activated during starvation in order to generate ATP. However, defective autophagy can cause cell death. Interference in the Ca^{2+} homeostasis will also interfere with cardiac contraction. Increased intracellular Ca^{2+} concentration in cardiac cardiomyocytes will activate the Ca^{2+}-activated protease, calpain, which in turn will degrade the cardiac proteins. Up to thirty five percent of protein synthesis occurs at the ribosomes associated with the SR, called the rough SR. Any damage or alteration to the SR will influence protein synthesis and SR stress will lead to misfolded, dysfunctional proteins (Chang *et al*., 1987).

2.14 Objectives

The objectives of this study were:

1. To investigate the mode of cell death (apoptosis, autophagy and necrosis) caused by exposure of H9c2 cells to pavetamine.
2. To perform a transmission electron microscopy (TEM) study of H9c2 cells exposed to pavetamine.
3. To conduct mitochondrial studies in H9c2 cells evaluating the:
   a. Mitochondrial membrane potential.
   b. Cytochrome c release.
   c. Cyclosporin A inhibition of the mitochondrial permeability transition pore (MPTP).
4. To study the effect of pavetamine on the subcellular organelles of H9c2 cells with fluorescent probes.
5. To label rat neonatal cardiomyocytes (RNCM) with antibodies to some contractile and cytoskeleton proteins.