1 Introduction

1.1 Compartmentalization of cellular signalling

Compartmentalization of cellular signaling is essential for complex interactions, which occur simultaneously between cells and sub-cellular compartments.

Membranes isolate cells and sub-cellular compartments, a prerequisite for the formation of organized structures including cells, tissues, organs and organisms. Intracellular compartmentalization creates different biochemical conditions inside one cell allowing carrying out essential activities at the same time (e.g. signal transduction, metabolic and catabolic and anabolic processes).

Beside compartmentalization by membranes, cells are able to create focal points of enzyme activity that organize the intracellular action of many hormones and neurotransmitters by creating multiprotein signaling networks. Anchoring proteins provide a molecular framework that orients these enzymes towards selected substrates. The local activation of the cyclic adenosine 3’5’-monophosphate (cAMP) dependent protein kinase (PKA) for example, is regulated by A-kinase anchoring proteins (AKAPs) (Wong and Scott, 2004).

1.2 Cyclic adenosine 3’5’-monophosphate (cAMP) mediated signalling

cAMP has been implicated in the mediation of many cellular processes such as certain metabolic events, muscle contraction, cytokine-inflammatory events, and differentiation and growth (Houslay and Milligan, 1997). Cells respond to extracellular stimuli, such as hormones and neurotransmitters. The extracellular signal is propagated to its target *via* a defined signal transduction pathway. Second messengers such as Ca2+ and cAMP regulate signal transmission.

The synthesis and release of cAMP into the cytoplasm is initiated by the occupancy of G-protein coupled receptors (GPCR) at the plasma membrane by receptor specific ligands. The ligand-bound GPCR catalyses the exchange of GDP for GTP on the α-subunit of the heterotrimeric G-protein, which results in the activation of the α-subunit and its dissociation from the β, γ-subunits. Both, the α-subunit or the β, γ-subunits can than initiate or inhibit distinct intracellular

signaling cascades. The α-subunit of the G_s subtype activates adenylyl cyclases (AC), which convert ATP to cAMP (Wong and Scott, 2004). Currently, nine different AC isoforms are known, which are mostly tissue-specific and regulated by different G-protein subunits (Cooper, 2003).

cAMP exerts its intracellular effects mainly by activating PKA. AKAPs bind both PKA and its substrates (Wong and Scott, 2004). However, cAMP can also act in a PKA-independent manner *via* the direct activation of ion channels or of Epacs (exchange protein activated by cAMP) (Rehmann et al., 2007). Members of the phosphodiesterase (PDE) family locally degrade cAMP into 5'-adenosine monophosphate (5'AMP) and thereby limit the stimulatory effect of the cAMP cascade, (Fig. 1.1).

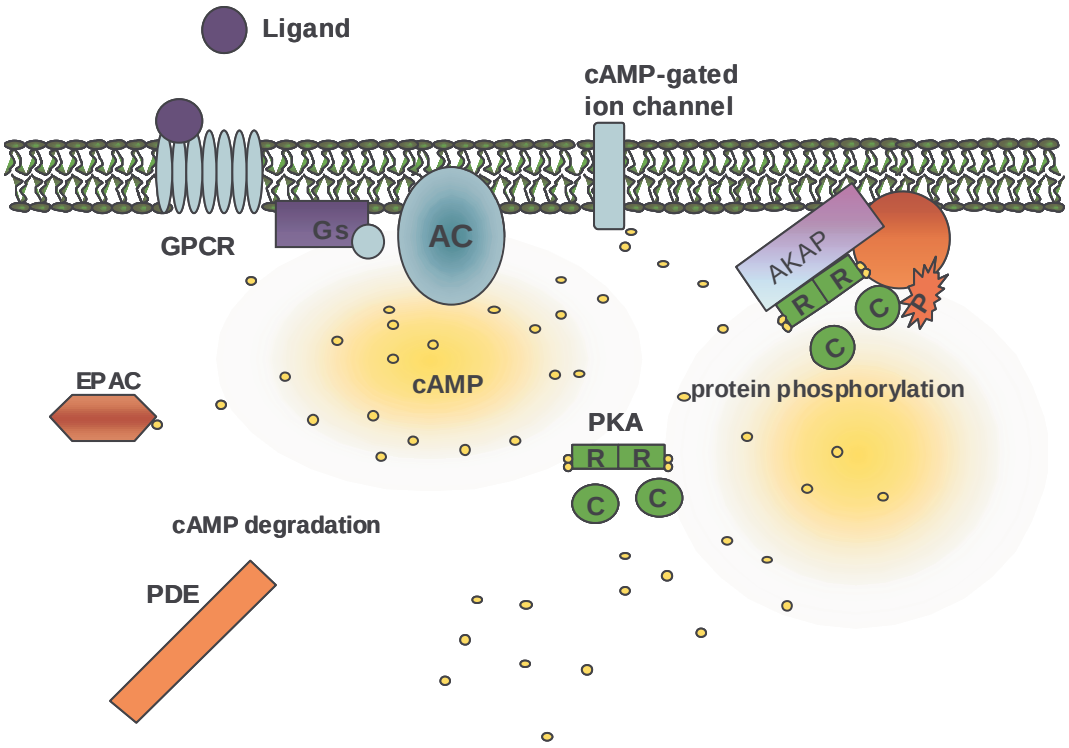

Figure 1.1 Initiation of cAMP signaling by G-protein coupled receptors. Ligands bind to the G-protein-coupled receptor (GPCR) causing the dissociation of the $G_{s\alpha}$ subunit. The $G_{s\alpha}$ subunit can stimulate adenylyl cyclase (AC) to produce cyclic AMP (cAMP). cAMP exerts its cellular effects *via* the activation of protein kinase A (PKA), Epac, or cyclic nucleotide gated ion channels (Wong and Scott, 2004).

1.3 Protein kinase A (PKA)

The cAMP-dependent protein kinase (PKA) has a broad substrate specificity (Skalhegg and Tasken, 2000; Shabb, 2001) and consists of two catalytic (C) subunits bound to a regulatory (R) subunit dimer. Three C-subunit genes (Cα, Cβ, and Cγ) have been characterized, and four R-subunit genes have been identified (RIα/β and RIIα/β). Depending of the presence of RI or RII subunits, PKA is designated type I or type II. Two molecules of cAMP bind to each R-subunit and induce the dissociation of the C-subunits, which phosphorylate their substrates (Fig.1.2). PKA is a serin/threonine kinase which catalyses the transfer of the γ-phosphate of adenosine triphosphate (ATP) to serine or threonine residues, which are embedded in a consensus motif of R-R-Ø-S (Ø: hydrophobic).

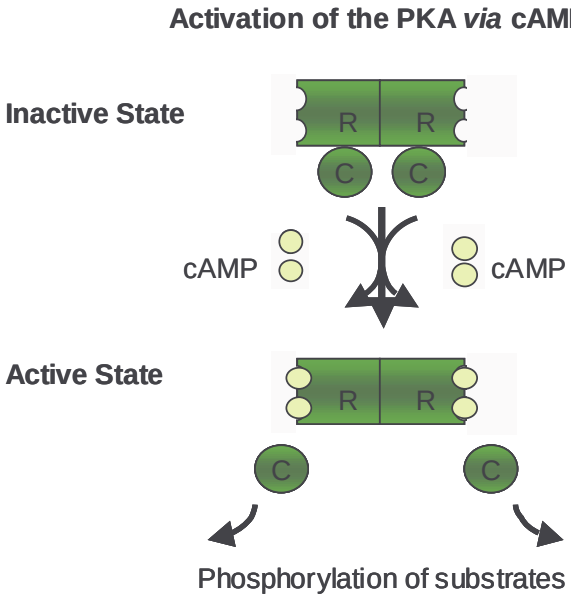

Figure 1.2 Activation of PKA. Binding of two molecules of cAMP to the regulatory (R) subunits of PKA induces the dissociation of the catalytic (C) subunits.

The biological significance of the presence of two major isozymes, PKA RI and RII, is still uncertain. RI is predominant in many cells with rapid proliferation, rapid growth in cell size, and specially RIα expression is increased in a variety of carcinoma cells (Gupte et al., 2005). RIα knock out mice die in embryonic life, due to a deficit in the morphogenesis of the mesodermal embryonic germ layer (Amieux and McKnight, 2002), whereas RIIβ knock out, RIIα knock-out, and RIβ

knock-out mice have less obvious defects, mainly in differentiation of adipose tissue in the case for RIIβ (Cummings et al., 1996), and neural functions for RIβ (Brandon et al., 1995). In RII knock out mice, RIα may compensate for the loss of RII subunits (Amieux et al., 2002). RI is predominantly found in the cytosol whereas RII is mostly membrane-localized.

1.4 Phosphodiesterases

Cyclic nucleotide phosphodiesterases (PDEs) are a super family of enzymes, which hydrolyse both cAMP and cGMP. To date, the PDE super family includes 21 different genes encoding over 30 different PDEs, having different enzymatic characteristics, different regulatory properties and differential inhibitory pharmacological profiles (Conti and Jin, 1999; Houslay and Milligan, 1997; Houslay and Adams, 2003; Soderling and Beavo, 2000; Omori and Kotera, 2007). The ability of PDEs to interact with AKAPs provides a control for the PDE phosphorylation by PKA. PDEs have been shown to bind to several different AKAPs (Dodge-Kafka et al., 2006; Kapiloff, 2002; Tasken and Aandahl, 2004; Stefan et al., 2007).

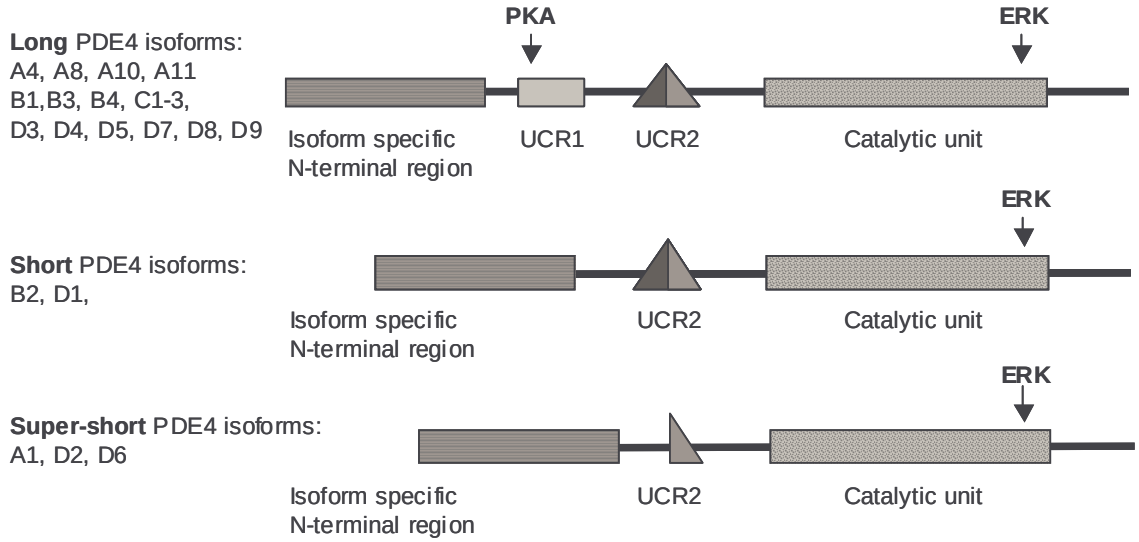

Figure 1.3 Schematic representation of the PDE4 family. PDE4D3 belongs to the long isoform and possess both upstream conserved regions (UCR), UCR1 and UCR2.

1.4.1 PDE4D3

The PDE4 phosphodiesterase family exclusively hydrolyses cAMP. PDE4D3 is the long form of the PDE4 family and contains two regulatory domains termed Upstream Conserved Regions 1 (UCR1) and 2 (UCR2), appearing to control the highly conserved catalytic region (Fig. 1.6). PDE4D3 is regulated by PKA and ERK phosphorylation (Terry et al., 2003; Baillie et al., 2005).

1.5 A-kinase anchoring proteins (AKAPs)

PKA localizes to discrete subcellular sites by interaction of the R subunits with AKAPs. Up to now, more than 70 different AKAPs have been identified in a variety of cell types. AKAPs are a divers group of proteins, varying in molecular weight and without obvious sequence homology. Each AKAP shows specificity for different binding partners and anchoring to subcellular compartments. The AKAP nomenclature generally uses the apparent molecular weight to denote each anchoring protein, e.g. AKAP18α has a molecular weight of 18 kDa. However, this nomenclature is irritating as there are several splice variants of the same AKAP. Furthermore, some anchoring proteins, such as gravin or Rab32, were named before it was realized that they bind PKA. Therefore, a new nomenclature was established by the Gene Nomenclature Comitee according numbers starting with 1 to each family of isoforms.

AKAP function currently incorporates three concepts: First, a protein is classified as an AKAP if it directly binds the R subunit of PKA (Fig.1.3). AKAPs contain an amphipathic α-helix of 14-18 amino acids, which binds to the N-terminal docking and dimerization domain of the R subunit dimer of PKA (Fig.1.4) (Newlon et al., 1997; Newlon et al., 2001; Hausken et al., 1996). Binding affinities for RII subunits differ greatly within the AKAP family (Alto et al., 2003; Herberg et al., 2000; Dransfield et al., 1997; Gronholm et al., 2003; Hundsrucker et al., 2006; Henn et al., 2004). Although most AKAPs bind to RII subunits, several AKAPs interact specifically with RI. D-AKAP1 and D-AKAP2 are examples for dual specificity AKAPs which anchor both types of R subunits (Angelo and Rubin, 1998; Huang et al., 1997b). In the case for D-AKAP1, RI binds with a 100 fold lower affinity to the AKAP than RII (Herberg et al., 2000).

Second, each AKAP possesses targeting domains for protein/protein or protein/lipid interactions, directing it to its specific subcellular localization. Several AKAPs are targeted to the same subcellular compartment, and splice variants from one AKAP may be differentially targeted (Wong and Scott, 2004). The α and β isoforms of AKAP18 are recruited to membranes trough myristoyl and dual palmitoyl groups (Trotter et al., 1999). In mouse oocytes the γ isoform of AKAP18 is targeted to the nucleus (Brown et al., 2003). AKAP18δ is targeted to intracellular vesicles and to the plasma membrane in renal principal cells through an unknown mechanism (Henn et al., 2004).

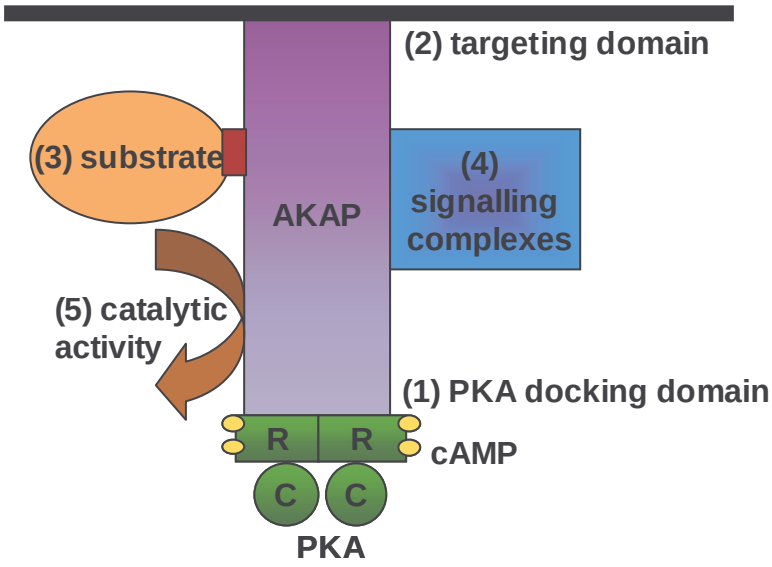

Figure 1.4 General schematics of AKAP interaction and function. (1) Docking and dimerization domain for PKA (2) targeting domain which directs the AKAP to distinct subcellular regions (3) PKA substrate (4) other binding partners (5) some AKAPs have own catalytic activity.

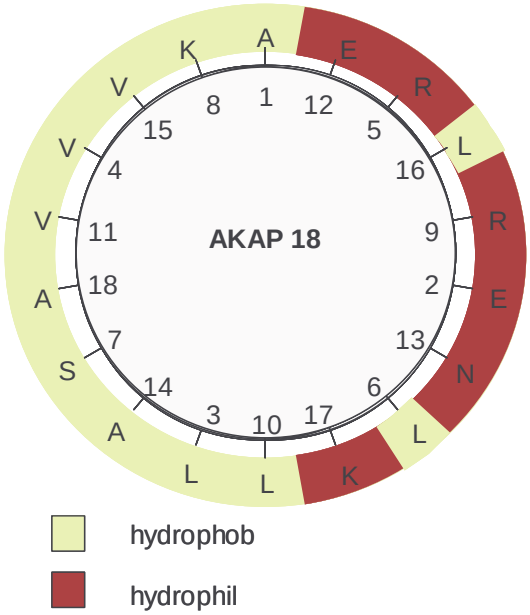

Figure 1.5 The RII binding domain of AKAPs forms an amphiphatical helix. Hydrophobic and hydrophilic amino acids are situated on contrary sites of the helix. As example the amphipatical helix of AKAP18 is shown.

Third, AKAPs possess is the ability to coordinate multiple signaling pathways by anchoring additional signaling enzymes such as phosphatases, phosphodiesterases (PDE), and other kinases (Fig.1.3), integrating signals from different signaling pathways in one complex. mAKAP combines cAMP dependent signaling with the ERK dependent signaling pathway by binding PKA and also PDE4D3, which tethers ERK5 to the complex (Dodge-Kafka et al., 2005). In the complex formed by the AKAP gravin and the β_2 adrenergic receptor, PKA and PKC can phosphorylate the same substrate. PKA mediated phosphorylation of gravin strengthens the interaction between the AKAP and the receptor. PKC-mediated phosphorylation of gravin causes it to dissociate from the receptor (Lin et al., 2000; Tao et al., 2003). AKAP-Lbc binds three protein kinases: PKA, PKC and PKD, and function itself as a guanine exchange factor for the small GTPase RhoA (Baisamy et al., 2005; Carnegie et al., 2004; Klussmann et al., 2001; Diviani et al., 2004).These complexes often include enzymes for both signal transduction and termination, allowing a negative feedback control for a given signaling process, as described for mAKAP in Fig.1.6, (Wong and Scott, 2004). The PDE4D3-mAKAP-PKA complex provides

a self-regulatory module. When cAMP levels rise, PKA bound to mAKAP is activated and phosphorylates and activates PDE4D3. As the activity of PDE4D3 increases rapidly following PKA phosphorylation, cAMP levels decrease and thereby decrease mAKAP-bound PKA activity, and PDE4D3 itself becomes dephosphorylated and less active (Terry et al., 2003). PKA phosphorylation of PDE4D3 also enhances its binding to mAKAP (Carlisle Michel et al., 2004; Ruehr et al., 2003). PDE4D3 also interacts with β-arrestins and this complex can be translocated from the cytosol to the β_2-adrenergic receptor (Baillie et al., 2003).

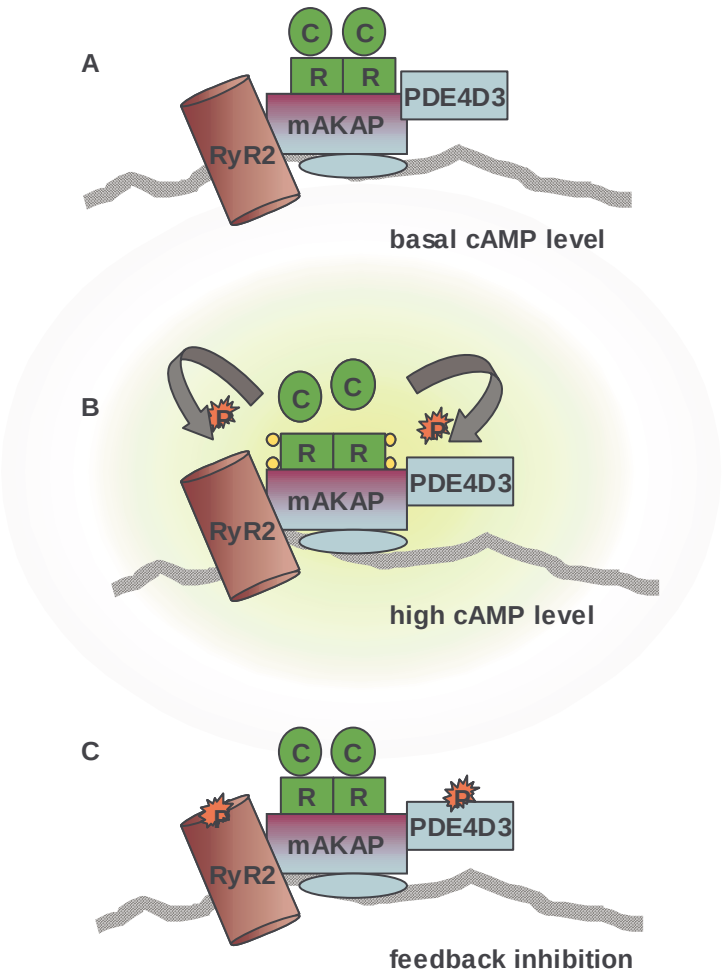

Figure 1.6 Negative feedback inhibition of mAKAP bound PKA by PDE4D3. Under resting conditions PDE keeps cAMP levels low and PKA inactive, A. Stimulation increases cAMP, allowing activation of PKA and phosphorylation of RyR2 and PDE4D3, B. Phosphorylation of PDE4D3 increases its activity for cAMP hydrolysis and its association to mAKAP. Phosphorylation induces a negative feedback loop to turn off PKA activity

1.5.1 The AKAP18 family

The AKAP18 family consists of four known splice variants, AKAP18α, β, γ and δ (Fig.1.7). Function of the AKAP18α complex in the heart is described in chapter 1.6.9, and function of AKAP18δ in the kidney is described in chapter 1.7.3.

AKAP18α was independently cloned from skeletal muscle and cardiac fibroblasts in two separate laboratories and was named after its apparent molecular weight. It was estimated to have a size of 15 kDa by Catterall and colleagues and thus was named AKAP15 (Gray et al., 1997). Scott and colleagues predicted the size to be 18 kDa and named it AKAP18 (Fraser et al., 1998). The initially found 81 amino acid AKAP 18/15 was renamed AKAP18α (Trotter et al., 1999).

AKAP18β and AKAP18γ were cloned from a human lung library by Trotter et al. AKAP18β consists of 104 amino acid residues. AKAP18α and β share a common membrane targeting domain, consisting of the N-terminal residues Gly-1, Cys-4, and Cys-5, modified by myristoylation and dual palmitoylation, respectively. Compared to AKAP18α, AKAP18β possesses an additional 23 amino acid domain, binding to the apical plasma membrane in polarized epithelial cells (Trotter et al., 1999).

AKAP18γ consists of 326 amino acid residues. Its distribution is so far investigated in mouse oocytes and cellular fractions derived from rat kidney. In the kidney it is present in the particulate and in the soluble fractions. In mouse oocytes, AKAP18γ targets PKA-RI subunits to the nucleus, suggesting an involvement in transcriptional regulation (Brown et al., 2003).

AKAP18δ was identified by Henn et al (Henn et al., 2004). It is a protein of 353 amino acids and was first isolated from principal cells of kidney IMCD. AKAP18δ lacks the membrane-targeting domain present in AKAP18α and AKAP18β, and palmitoylation is not detected. Sequence analysis suggests the presence of a myristoylation consensus site (Henn et al., 2004) and a leucine zipper motif. AKAP18δ is a high affinity AKAP. The K_d values for the binding of

AKAP18δ to RIIα and RIIβ subunits of PKA are 31 and 20 nM respectively (Henn et al., 2004).

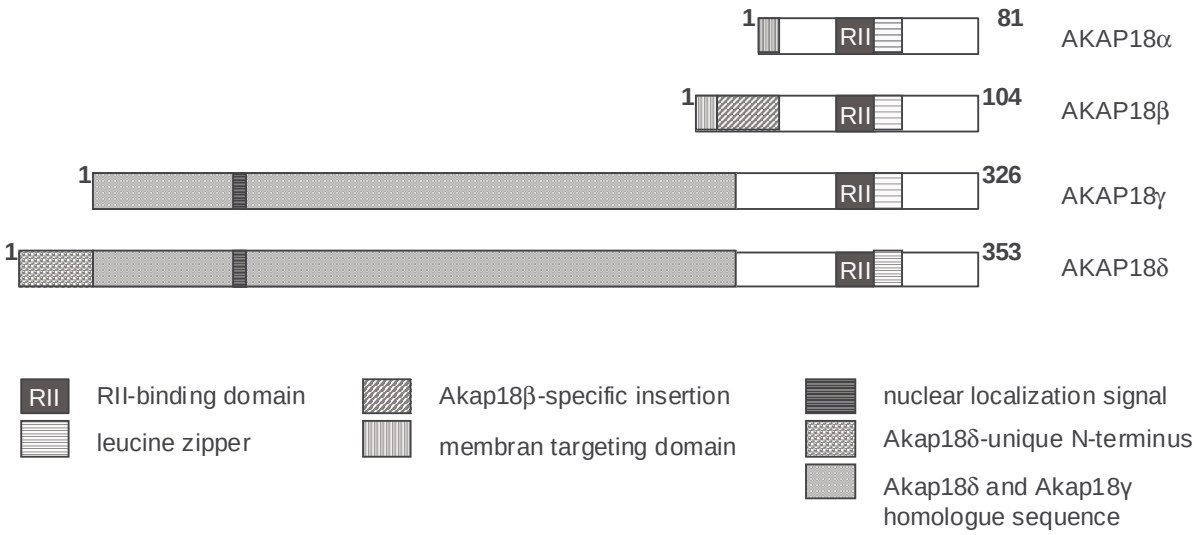

Figure 1.7 Schematic representation of AKAP18 isoforms. Amino acid residues are indicated by numbers. The membrane targeting domains of AKAP18α and β (vertical stripes) and the RII-binding sites (RII) of all isoforms are identical. Amino acids 1–245 of AKAP18γ and 27–272 of AKAP18δ are homologous (grey). The N terminus of AKAP18δ (amino acids 1–26, horizontal stripes) is unique to this isoform.

1.6 Compartmentalization of cellular signaling in cardiac myocytes

1.6.1 Organization of the cardiac myocyte

The cardiac muscle is composed of interconnected mono-nucleated cells, the cardiac myocytes. Each cardiac myocyte contains a large number of myofibrils. Each myofibril is separated by Z-discs into sarcomers. Actin filaments are fixed on the Z-discs. Myosin filaments interdigitate the actin filaments. In the middle of each sarcomere lies the M-disc (Fig.1.8). A large fraction of the cell volume is occupied by mitochondria, which synthesise ATP to supply energy for the constantly working heart muscle. Myofibrils and mitochondria occupy about 85% of the myocyte volume, the rest is composed of the transverse tubules (T-tubules), the sarcoplasmic reticulum (SR) and the cytoplasm (sarcoplasm).

Cardiac myocytes are surrounded by a membrane with unique properties called sarcolemma. Intercalated disks, which connect adjacent cardiac myocytes, and gap junctions, which form contact sites between the plasma membranes of adjacent cells, allow the generation and conduction of an electrical signal, the action potential (AP), through the heart leading to near-synchronous depolarisation of myocytes. In addition, the sarcolemma possesses the protein machineries necessary for the initiation of the excitation-contraction coupling process.

A system of T-tubules extends the sarcolemma into the interior of cardiac myocytes. T-tubules are perpendicular to the sarcomere at the level of the Z-disc, or may run longitudinally along the sarcomere. By extending the extracellular space into the cell, the electrical excitation of the sarcolemma can be brought into close proximity of the contractile proteins, enabling more rapid contraction and relaxation. The T-tubule system contains a large number of Ca^{2+} channels taking part in the ß-adrenergic regulation of the myocyte (Fig.1.9 and 1.10).

The SR is a tubular, membranous network within muscle cells, forming a close system without connection to the intra- or extracellular space. The SR is the equivalent of the endoplasmic reticulum in other cells and functions as the

intracellular Ca^{2+} store. It surrounds the myofibrils by a tubular system and connects them with the sarcolemma. The SR forms a terminal cistern in close proximity to the T-tubules, this site being called in the heart a diade (Fig.1.9 and 1.8) (Silbernagl and Despopoulos, 2003; Stefan et al., 2007).

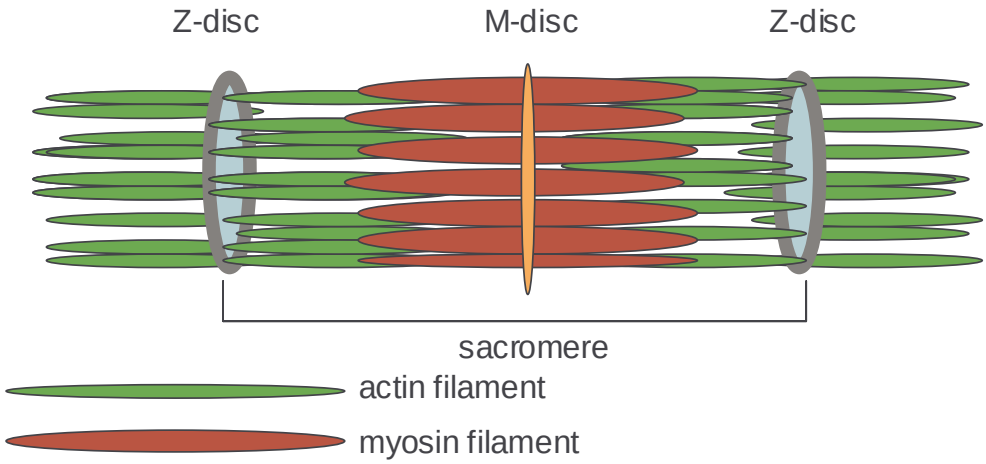

Figure 1.8 Structure of the sarcomere. Myofibrils consist of a row of sarcomeres. Each sarcomere contains interdigitating thin actin and thick myosin filaments. Actin filaments are fixed on the z-discs.

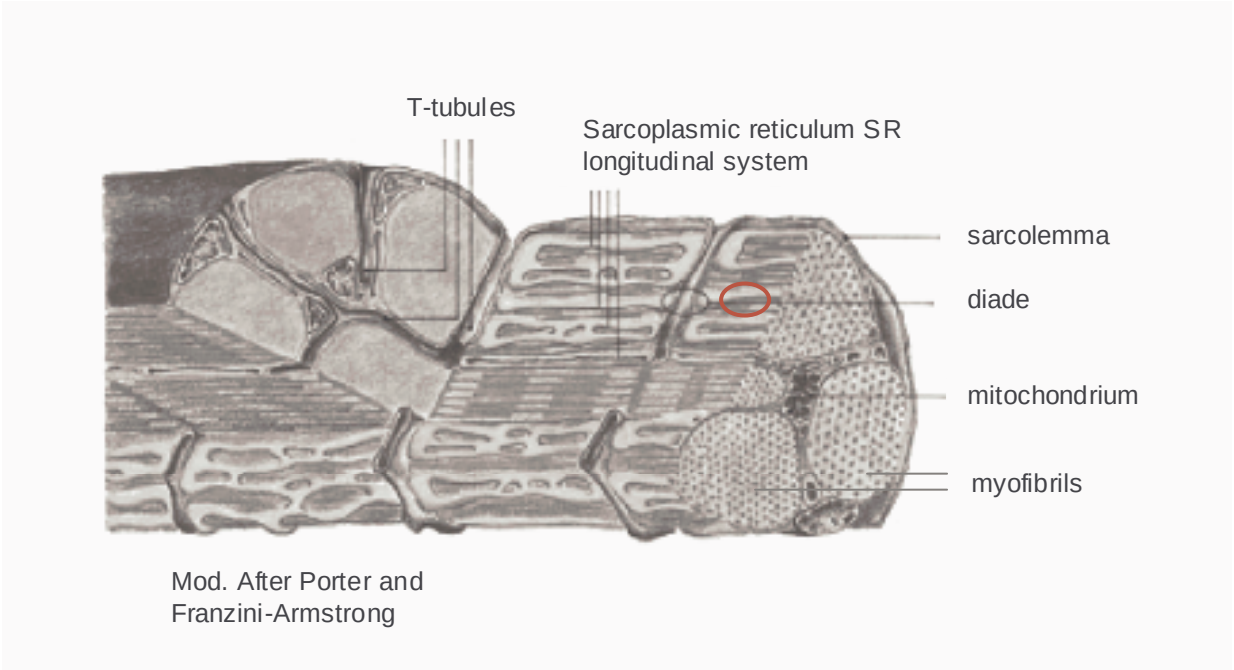

Figure 1.9 The sarcomeric system. The transversal (T)-tubules are invaginations of the plasma membrane. They enter into each myocyte at the level of the Z-disc and allow for a close contact between the plasma membrane and the sarcoplasmic reticulum (SR), forming the diade.

1.6.2 Molecular mechanism of contractility

Contraction and relaxation in cardiac myocytes are regulated by Ca^{2+} cycling between the sarcoplasm and the SR. Rhythmic heart muscle contraction is induced by action potentials (AP) generated by pacemaker cells located in the sinus and other centres within the heart muscle, initiating depolarization of the plasma membrane. Depolarization from –90 mV to greater than +40 mV starts with inward Na^{+} channel currents and is maintained by inward Ca^{2+} channel currents (Suzuki et al., 2002). The action potentials induce opening of L-type Ca^{2+} channels, also called dihydropyridine receptors (DHPR) located in the sarcolemma on the T-tubules. This triggers an influx of Ca^{2+} into the sarcoplasm of cardiac myocytes. The rise in cytoplasmic Ca^{2+} concentration activates the Ryanodine receptor type2 (RyR2), a Ca^{2+} activated Ca^{2+} release channel located in the SR membrane at the terminal cistern. The close proximity between RyR2 and L-type Ca^{2+} channels at the diade makes possible the fast response to the activating Ca^{2+} release (spark). Activation of RyR2 by the Ca^{2+} spark induces the release of Ca^{2+} from the SR into the sarcoplasm, (Fig.1.10). The combination of Ca^{2+} influx and release increases the free intracellular Ca^{2+} concentration, allowing Ca^{2+} to bind to the myofilament protein troponin C and causing its conformational change, which removes a steric block to the interaction between actin in thin filaments and myosin in thick filaments, allowing myocardium contraction. Cardiac relaxation requires removal of Ca^{2+} from the cytoplasm. Cytoplasmic Ca^{2+} is mainly transported into the SR through the ATP-dependent Ca^{2+} pump SERCA2, and out of the cell through the sarcolemmal Na^{+}/Ca^{2+} exchanger. SERCA2 activity is under tight control of the SR membrane protein phospholamban (PLB). In its dephosphorylated state, PLB inhibits SERCA2, while phosphorylation of PLB releases this inhibition, allowing a faster decline of cytoplasmic Ca^{2+} concentration and faster muscle relaxation, (see Fig.1.11) (Lygren and Tasken, 2006; MacLennan and Kranias, 2003).

Diastolic Ca^{2+} concentration in the cytosol is around 0.01 µM, and can raise up to 1 µM for contraction activation. In the SR, Ca^{2+} concentration is in the millimolar range resulting in a 10, 000 fold gradient across the SR-membrane. This concentration gradient leads to a flow of around 3 million ions per second

per RyR2. SERCA2, on the other hand, has been estimated to pump 30 ions per second (Katz, 1992; Stefan et al., 2007).

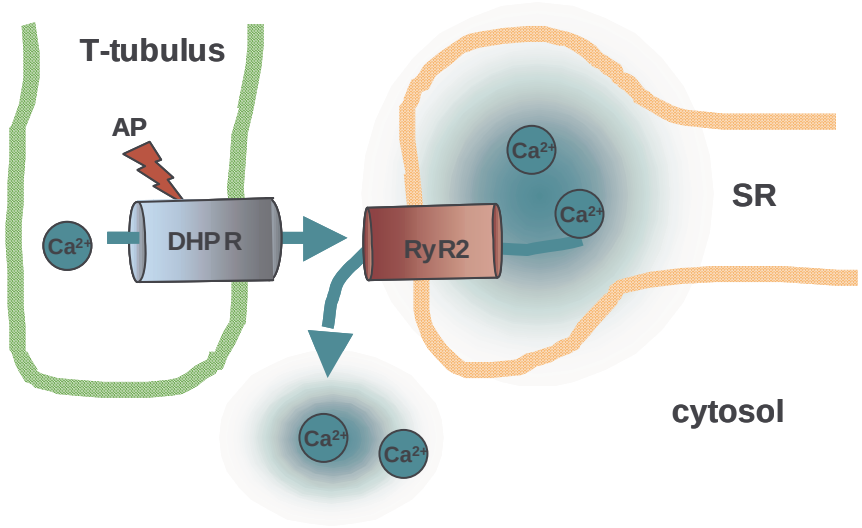

Figure 1.10 Diade in the myocyte. The close proximity between Ca^{2+} receptors in the plasma membrane and the SR at the level of the diade allows the release of Ca^{2+} from the SR after an action potential reaches the t-tubule.

1.6.3 Adrenergic stimulation

The intracellular Ca^{2+} concentration in the myocyte is not only determined by depolarization, but can be influenced by the catecholamines adrenaline and noradrenalin. These hormones are released after stimuli. They activate α and β adrenergic receptors (AR) on the surface of the myocytes and increase the heart contraction. Stimulation of the α- adrenergic system activates the inositol-phospholipid second messenger system and leads to an increased release of Ca^{2+} out of the SR. Stimulation of the β-adrenergic system activates the cAMP second messenger system and leads to an increased Ca^{2+} flux through the sarcolemma (Eckerdt, 2000).

β- adrenergic receptors (β-AR) act by coupling to the stimulatory G_s and inhibitory G_i proteins. The β-AR are divided into 3 subtypes (β1, β2, and β3), and additionally a fourth subtype that has not been well characterized yet. The β1-AR is the dominant subtype in the heart, representing 70-80% of β-ARs

(Hein, 2001). Both β1 and β2 subtypes are coupled to G_s protein, β2-AR can also bind to G_i. (Biolo et al., 2006).

Stimulation of the β-AR activates G_s and subsequently the adenylate cyclase (AC) and cAMP levels increase, activating PKA (Fig.1.11). The cAMP second messenger system regulates the inotropic (contraction force) and chronotropic (heart rate) cardiac response. PKA phosphorylation of the DHPR and of RyR2 increases the open probability of the channels increasing the Ca^{2+} transport into the cytosol and thereby muscle contraction (Marx et al., 2000; Gao et al., 1997). PKA phosphorylation of PLB induces the Ca^{2+} re-uptake into the SR and thereby muscle relaxation (Asahi et al., 2000).

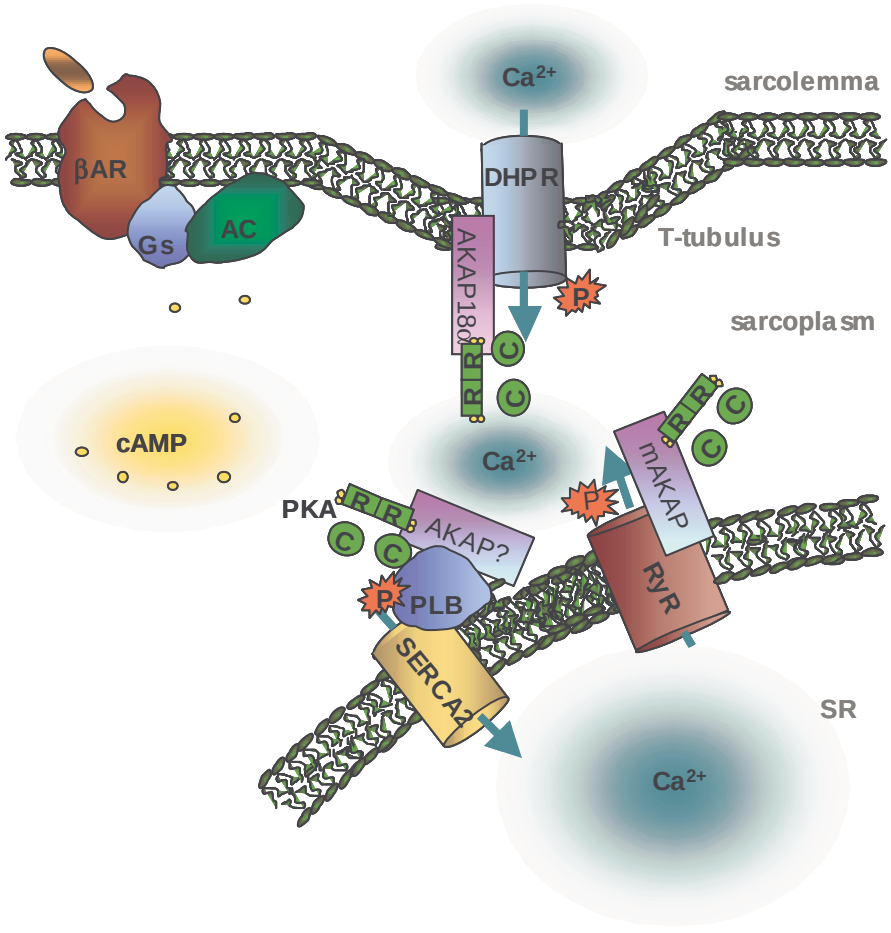

Figure 1.11 The interaction of the cAMP and Ca^{2+} pathways in cardiac myocytes after ß-adrenergic stimulation. Major components of the excitation/contraction coupling in the myocyte are shown. The cycling of Ca^{2+} is indicated by arrows. β-adrenergic receptor (β-AR), stimulatory G-protein (G_s), adenylyl cyclase (AC), sarcoplasmic reticulum (SR), ryanodine receptor (RyR2), ATP-dependent Ca^{2+} pump (SERCA2), phospholamban (PLB), L-type Ca^{2+} channel (DHPR).

1.6.4 Molecular regulation of the PLB-SERCA system

Ca^{2+} re-uptake into the cardiac SR is regulated primarily by the inhibitory effect of PLB on SERCA2. The ATPase activity of SERCA2 accounts for a significant part of the total heart energy consumption and a role for PLB is therefore to limit SERCA energy consumption in the resting state.

1.6.5 Sarcoplasmic Ca^{2+}-ATPase (SERCA2)

MacLennan first purified SERCA from detergent-solubilized SR vesicles from rabbit skeletal muscle in 1970 (MACLENNA.DH, 1970). Three different isoforms exist (SERCA1, 2 and 3) with two different transcript forms (a and b) for SERCA1 and 2. SERCA2a is the specialized sarcomeric isoform expressed to high levels in cardiac myocytes and slow-twitch skeletal muscles and to a lower extend in some types of smooth muscle and neuronal cells (Zarain-Herzberg et al., 1990). SERCA2a is a 110 kDa transmembrane protein, containing 10 membrane-spanning segments, and a cytoplasmic globular domain. This cytoplasmic domain contains the site of catalytic phosphorylation by ATP, and those residues predicted to comprise the nucleotide binding site, as well as the residues of the PLB binding site. Several conformational steps have been identified, which are coupled with translocation of Ca^{2+} across the SR membrane, involving the formation of a phosphoenzyme intermediate, energized by ATP (Chen et al., 2003; Mueller et al., 2004).

1.6.6 Phospholamban (PLB)

Only one PLB gene has been found. PLB is expressed in cardiac, slow-twitch skeletal and smooth muscle. PLB was first identified as a substrate for PKA in canine cardiac muscle SR, and was postulated to function as a regulatory factor of SERCA (Kirchberber et al., 1975; Morkin and LaRaia, 1974). It is a pentamer of identical subunits (Jones et al., 1985), which accounts for the original observation that it has an apparent mass of 22 kDa. Each monomer consists of 52 amino acid residues with a molecular weight of 6,1 kDa, and is organized in 3 physical and functional domains (see Fig.1.12): The cytosolic domain is composed of two helices, helice Ia including amino acids 1-16 and helice Ib including amino acids 21-30. Helice Ia and Ib are connected by a short ß-turn, serving as a flexible linker (Pantano and Carafoli, 2006; Metcalfe et al., 2004;

Metcalfe et al., 2004; Zamoon et al., 2005).The third part of the monomer is the transmembrane domain II (amino acids 31-52). The cytosolic helix Ia and the linker region contain the PKA-phosphorylation site serine (Ser) 16, and the Calmoduline kinase (CaMKII)-phosphorylation site threonine (Thr) 17. Cytosolic helix Ib is rich in glutamate and aspartate. Two helical faces can be distinguished on the transmembrane domain. One face of the helix contains residues involved in the PLB-SERCA interaction, at the other face, residues important for the formation of pentamers are situated (Kimura et al., 1997; Metcalfe et al., 2004). Mutagenesis studies reveal that SERCA binds to residues in the cytosolic part of PLB (Chen et al., 2006; Metcalfe et al., 2004; Metcalfe et al., 2004; Toyofuku et al., 1993), but the inhibitory function is retained in PLB molecules in which the cytosolic domains are largely deleted, showing that PLB-SERCA transmembrane interactions have a key inhibitory role. The PLB monomer is the active, SERCA inhibiting form, whereas the PLB pentamer must function as an inactive or less active reservoir.

1.6.7 Regulatory features of the PLB-SERCA interaction

James et al. (James et al., 1989; Metcalfe et al., 2004) provided the first clear evidence of a physical interaction between PLB and SERCA. Two steps can be distinguished in the reversible inhibition of SERCA activity by PLB: The association/dissociation of pentameric PLB (K_{d1}), and the association/dissociation of monomeric PLB and SERCA (K_{d2}) (Kimura et al., 1997). The phosphorylation of PLB, or Ca^{2+}-binding to SERCA, reverses SERCA inhibition (Asahi et al., 2000). In its unphosphorylated form, PLB inhibits SERCA function because of its interaction in the transmembrane domain (Zamoon et al., 2005). Many PLB mutations alter either one or both dissociation constants (K_{d1}, K_{d2}), leading to the gain or loss of inhibitory function (MacLennan and Kranias, 2003). NMR and EPR spectroscopy of the SERCA/PLB complex reveal that PLB undergoes allosteric activation upon encountering SERCA and structural changes after phosphorylation (Fig.1.12.B and Fig.1.13) (Metcalfe et al., 2004; Zamoon et al., 2005; Pantano and Carafoli, 2006).

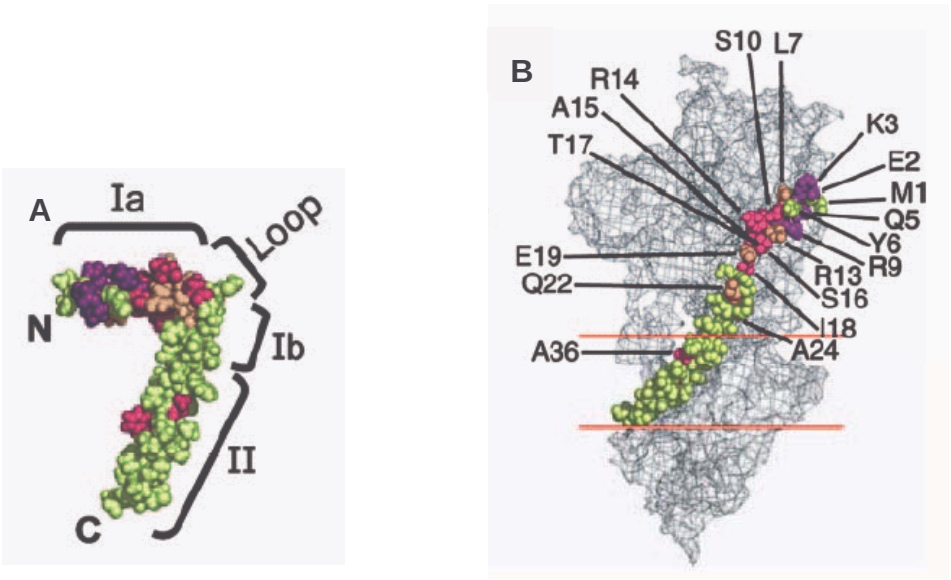

Figure 1.12 Mapping of interacting residues between SERCA and PLB onto PLB NMR structure. A Mapping of the interacting residues onto the computational model of the PLB-SERCA complex, **B**. Red lines indicate the boundary of the hydrophobic core of the lipid bilayer(Zamoon et al., 2005).

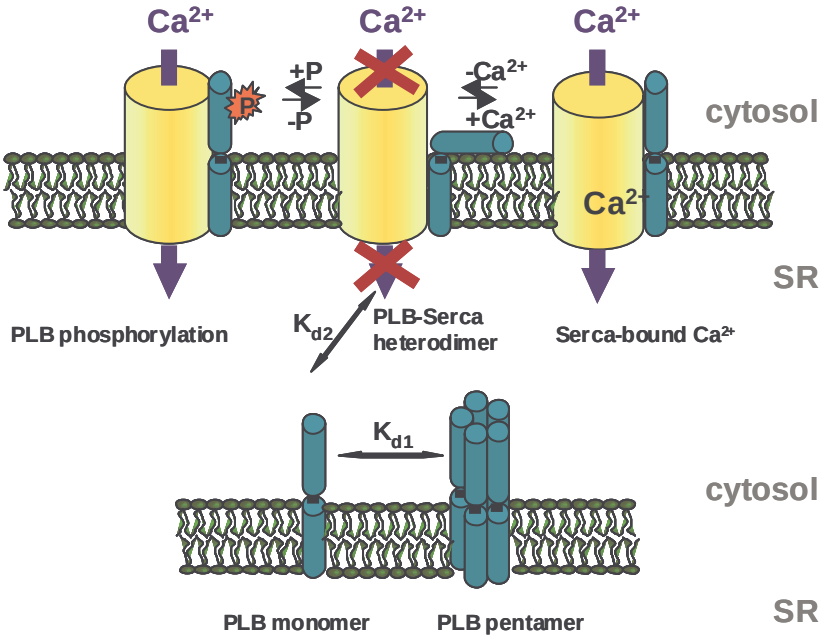

Figure 1.13 Regulatory features of the PLB-SERCA interaction. The reversible inhibition of SERCA by PLB consists of two steps: The association/dissociation of pentameric PLB (K_{d1}), and the association/dissociation of monomeric PLB and SERCA (K_{d2}), after PKA phosphorylation or Ca^{2+} binding to SERCA, PLB no longer inhibits SERCA function (Kimura, 1997; Zamoon, 2004).

1.6.8 PDEs in cardiac myocytes

A range of different PDE variants is expressed in cardiac myocytes. These include the PDE1, PDE2, PDE3, PDE4 and PDE5 families (Maurice et al., 2003). PDE3 and PDE4 families provide the major cAMP-hydrolysing phosphodiesterase activity in the heart (Mongillo et al., 2004; Takahashi et al., 2002). The enzymes of the PDE4 family provide more than twice the cAMP-hydrolysing activity of the PDE3 family. The PDE4D isoforms provide more than two thirds of the total PDE4 activity (Mongillo et al., 2004). PDE4D3 is targeted to the sarcomeric region through binding to an anchor protein called myomegalin (Verde et al., 2001) and to the RyR2 complex in the SR and the perinuclear region through binding to muscle-selective AKAP (mAKAP) (Dodge et al., 2001) (Ruehr et al., 2003).

PDE activity contributes to generate cAMP microdomains involved in the ß-adrenergic stimulation of Ca^{2+} channels (Fischmeister et al., 2006). In mouse neonatal myocytes, PDE4D was shown to selectively impact cAMP signaling by β_2-AR, while having little or no effect on β_1-AR signaling (Xiang et al., 2005).

1.6.9 AKAPs in cardiac myocytes

ß-adrenoreceptor stimulated activation of PKA by cAMP is responsible for the modulation of many cellular functions in the heart including contraction force and heart rate by modulating ion, especially Ca^{2+} fluxes. Disturbances in cAMP signaling can cause cardiac hypertrophy and heart failure. Several studies reveal, that a general increase in cAMP concentration is not sufficient for the specific regulation of proteins. For example, PKA must be colocalized with its targets to ensure spatial-temporal control of cAMP/PKA-dependent substrate phosphorylation. AKAPs play a central role by providing a high level of specificity. Several AKAPs are expressed in the heart, including AKAP18α described below, and are known to be involved in modulation of cardiac contraction coupling (Dodge-Kafka et al., 2006; Kapiloff, 2002; Ruehr et al., 2004).

The action potential in the contracting cardiac myocyte is initiated by the depolarization of the plasma membrane. The L-type Ca^{2+} channel determines the plateau phase of the action potential of the cardiac myocyte and its open probability is increased by PKA phosphorylation after β_2-AR stimulation (Bers

and Weber, 2002; Dodge-Kafka et al., 2006). AKAP18α is associated with the L-type Ca^{2+} channel and thereby plays a crucial role in ß-adrenoreceptor stimulation, (Fig. 1.9). AKAP18α is located exclusively at the sarcolemma *via* lipid anchoring of covalently attached lipid moieties. AKAP18α directly interacts with the α-subunit of the L-type Ca^{2+} channel *via* a leucine zipper motif near the C-terminus (amino acids 1774-1841) and the amino acids 25-54 of AKAP18α (Hulme et al., 2002). Regulation of the K^+-channel KCNQ1 controls the duration of the action potential in the heart. The AKAP Yotiao targets PP1, RII and C-subunit of PKA to this receptor, and phosphorylation at S27 by PKA increases channel currents (Marx et al., 2002).

An other AKAP expressed in the cardiac myocyte is AKAP79, which targets PKA to the $ß_2$-AR and, in addition tethers PKC and calcineurin (PP2B) to this complex. PDE4D5 interacts with the signaling scaffold protein β-arrestin and is thereby recruited to the $ß_2$-AR upon agonist challenge, integrating several second messenger-signaling systems and linking upstream activators with downstream substrates (Baillie et al., 2003; Coghlan et al., 1995; Klauck et al., 1996). Several other AKAPs are identified in adult cardiac myocytes, including ezrin, AKAP-lymphomablastoma crisis (Lbc), mAKAP and AKAP220. Tab.1 and gives an overview of AKAPs identified in the heart and of their probable localization in cardiac myocytes, as well as their associated proteins.

name	**Interaction partners**	**Localization in the myocyte**	**Other tissues**	**Reference**
AKAP-Lbc Ht31 (AKAP13)	PKA, PKC, PKD, RhoA, 14-3-3, $Gα_{12}$, $Gα_{13}$	actin stress fibers	Kidney	(Carnegie et al., 2004; Klussmann et al., 2001; Diviani et al., 2004)
AKAP15/18α, AKAP18β, γ, δ (AKAP7)	PKA, L-type Ca^{2+}-channel, $ß_2$-AR, PP2B, PKA, Na^+-channel	SR/T-tubules	kidney, brain, skeletal muscle	(Fraser et al., 1998; Gray et al., 1997; Potet et al., 2001; Tibbs et al., 1998)
mAKAP AKAP100 (AKAP6)	PKA, PDE4D3, Ryanodine receptor RyR2, PP1, PP2A, PP2B, nesprin-1α, MEK5, ERK5, EPAC1	SR, nuclear envelope	nuclear envelope of skeletal muscle and brain	(Bers, 2004; Dodge-Kafka and Kapiloff, 2006)

name	Interaction partners	Localization in the myocyte	Other tissues	Reference
AKAP79/75/150 (AKAP5)	PKA, ß$_2$-AR, PKC, PP2B, DHPR, GABA(A) receptor	plasma membrane	Brain, postsynaptic densities	(Brandon et al., 2003; Higashida et al., 2005; Sandoz et al., 2006)
Gravin/ AKAP250 (AKAP12)	PKA, PKC, PPA, PP2B, ß$_2$-AR, G-protein-linked receptor kinase-2, ß-arrestin, clathrin, actin, src	actin cytoskeleton	Epithelia, testis, kidney, skeletal muscle	(Tao et al., 2007; Wang et al., 2006)
Yotiao/ AKAP350/ 450/ CG-NAP (AKAP9)	PKA, PP1, KCNQ1, PDE4D3, casein kinase-1, PP2A, PKCε, PKN, NMDA	plasma membrane, centrosome	brain, pancreas, liver, lung, spleen, skeletal muscle, postsynaptic densities, neuromuscular junctions, centrosome and Golgi	(Lin et al., 1998; Chen et al., 2005; Schmidt et al., 1999; Tu et al., 2004; Witczak et al., 1999)
BIG2	PKA, Arf, AMY-1, GSK3β	Golgi, secretory vesicles	brain, kidney, placenta, pancreas	(Li et al., 2003)
AKAP220	PKA, PP1, GSK3β	Golgi, secretory vesicles	testis, brain	(Schillace et al., 2001; Tanji et al., 2002)
AKAP95	P68 RNA helicase, DNA, AMY-1 HCAP-D2/Eg7, fidgetin, PDE4A, cyclin D3, MCM2	nuclear matrix	T-lymphocytes, thyroid gland	(Arsenijevic et al., 2004; Furusawa et al., 2002; Yang et al., 2006; Asirvatham et al., 2004)
D-AKAP-1	PKA, PP1,	mitochondria and SR	adypocytes	(Bridges et al., 2006; Yang et al., 2006; Huang et al., 1997b)
AKAP121/S-AKAP84 (AKAP1)	PKA, PTEN, mRNA, protein tyrosine phosphatase D1, AMY-1	mitochondria and ER	skeletal muscle, testis, thyroid, kidney, liver	(Cardone et al., 2004; Feliciello et al., 1998; Huang et al., 2005; Sardanelli et al., 2006)
AKAP149	PKA, PDE4A, PP1, mRNA		testis, thyroid,	(Rogne et al., 2006; Steen et al., 2003)
D-AKAP-2 (AKAP10)	PKA, Gα		skeletal muscle	(Burns-Hamuro et al., 2004; Huang et al., 1997a; Russell et al., 2006)

name	Interaction partners	Localization in the myocyte	Other tissues	Reference
synemin	PKA, vinculin, α-distrobrevin, α-actinin	cytoskeleton		(Russell et al., 2006)
Sphingosine Kinase Interacting protein 1 (SKIP1)	PKA, sphingosine kinase	membrane		(Lacana et al., 2002; Scholten et al., 2006)
Ezrin (AKAP78)	PKA, actin, EBP50, NHERF2, CFTR, NHE3, $ß_2$-AR , adenosine 2a receptor	actin cytoskeleton	secretory epithelia	(Dransfield et al., 1997)

Table 1. AKAPs in cardiac myocytes. Listed are AKAPs known to be expressed in the heart, their potential binding partners and the cellular localization in the myocyte. In addition, cells/tissues other than myocytes are listed, known to express the corresponding AKAP.

1.6.10 PLB and AKAPs in heart failure

1.6.10.1 Alterations in PLB

Abnormal Ca^{2+} cycling in animal models of heart failure and human failing hearts reflects in part impaired SR Ca^{2+} re-uptake that results in permanent elevated cytosolic Ca^{2+} levels (Beuckelmann et al., 1992; Dipla et al., 1999). The amount of SERCA is decreased relative to PLB in the failing heart, leading to an increased inhibitory function of PLB and prolonged relaxation time. Consequently, the SR Ca^{2+} store decreases and less Ca^{2+} is available for subsequent contractions (Dash et al., 2001; Frank et al., 1998). These studies suggest that the PLB-SERCA interaction is an attractive target for therapeutic interventions to increase cardiac contractility. Recent studies show beneficial long-term effects on cardiac function by gene transfer of mutant PLB in BIO 14.6 hamster and in myocardial infarct rat (Hoshijima et al., 2002). Gene transfer of a PLB-binding antibody improves Ca^{2+} handling and cardiac function in the failing cardiomyopathic hamster heart (Dieterle et al., 2005), overcoming cardiac impairment by lowering SERCA inhibition. However, a large discrepancy between the cardiac phenotypes of reduced PLB expression in mice and humans exist. PLB ablation showing a beneficial effect on disturbed cardiac function in the animal model, is identified as a cause for dilated cardiomyopathy in humans (Haghighi et al., 2003).

Dilated cardiomyopathy segregates in a few human families, in some cases caused by mutations in the *PLB* gene. A human PLB mutation, Leu39stop is

discovered in two large Greek families (Haghighi et al., 2003). The Leu39stop mutant is unstable, or misrouted to other membranes and no PLB was detectable in the heart of homozygous individuals, which develop lethal cardiomyopathy.

The inheritance of the PLB mutation encoding Arg9Cys is linked to the dominant inheritance of dilated cardiomyopathy (Schmitt et al., 2003). In heterologous cell culture and a transgenic mouse model, PLB (Arg9Cys) binds subunits of PKA, which blocks PKA-mediated phosphorylation of wild-type PLB and in turn delays Ca^{2+} re-uptake into the SR.

1.6.10.2 Alterations of AKAPs in heart failure

Considering that AKAPs play an important role in modulating PKA dependent phosphorylation of proteins regulating cardiac contractility, altered AKAP expression or PKA-AKAP anchoring may be the reason for some heart diseases. It is reported that the expression of mAKAP, RyR2 and SERCA2a is decreased in *mdx* hearts, a mouse model of Duchenne muscular dystrophy (DMD), leading to abnormal intracellular Ca^{2+} homeostasis and muscle weakness (Rohman et al., 2003). The affinity of PKA for AKAP binding may also be altered in the failing heart. PKA is able to phosphorylate its own RII subunits (autophosphorylation). The level of autophosphorylated RII is decreased in failing heart (Zakhary et al., 2000b) and causes a change in RII affinity for several AKAPs expressed in the heart. RII autophosphorylation increases the affinity of RII for AKAP-Lbc, mAKAP or AKAP15/18 by 2-, 16-, or 250-fold, respectively, which may provide additional localized regulation of PKA anchoring (Zakhary et al., 2000a). It was proposed that AKAP79 plays a role in overcoming cardiac hypertrophy through its inhibiting effect on calcineurin (PP2B) (Fiedler and Wollert, 2004; Taigen et al., 2000; Yatani et al., 2001). The interaction between AKAP Yotiao and the K^+-channel KCNQ1 is likely to have important clinical implications. The Yotiao binding domain of KCNQ1 (leucine zipper motif) is mutated in individuals with long QT syndrome. This suggests that the disruption in the sympathetic regulation of action potential duration seen in these individuals may, in part, be regulated by the targeting of PKA to this K^+-channel via Yotiao (Terrenoire et al., 2005). The GTPase RhoA is involved in the signaling pathway leading to cardiac hypertrophy. AKAP-Lbc is critical for

RhoA activation and transduction of hypertrophyc signals. Suppression of AKAP-Lbc expression in rats reduces RhoA activation and hypertrophic responses (Appert-Collin et al., 2007).

1.7 PKA anchoring in the kidney

1.7.1 Water channel trafficking in kidney cells: Aquaporins and water permeability

In order to maintain water and osmolyte balance, the human kidney forms 180 litres of primary urine per day, which is reabsorbed through aquaporin water channels. Aquaporin (AQP)-1 is constitutively present in the apical and basolateral plasma membrane of proximal tubules and descending limbs of Henle (King et al., 1996). Upon exposure with the antidiuretic hormone arginine vasopressin (AVP), principal cells of the renal collecting duct concentrate the primary urine *via* AQP2 through the apical and *via* AQP3 and AQP4 through the basolateral plasma membrane(Agre and Nielsen, 1996; Marples et al., 1999; Nielsen and Agre, 1995; Nielsen, 1996).

1.7.2 PKA-triggered AQP2 trafficking

AVP is a hormone acting through a G-protein coupled receptor and activating the cAMP-PKA signaling pathway. Binding of AVP to the V2 receptor, located in the basolateral plasma membrane of renal collecting duct principal cells, results in the redistribution of AQP2 from intracellular vesicles to the plasma membrane (AQP2 redistribution; (Klussmann et al., 2000; Nielsen et al., 2002; Valenti et al., 2005; King et al., 2004). This process constitutes the molecular basis of AVP-stimulated water reabsorption, (see Fig.1.14). Inactivating mutations in the V2 receptor or *AQP2* gene causes nephrogenic diabetes insipidus (NDI), a disease characterized by a massive loss of water (Morello and Bichet, 2001). AVP stimulates the elevation of cAMP followed by activation of PKA. PKA phosphorylates AQP2 at serine 256 (S256). AQP2 contains potential consensus sites for at least five kinases, namely PKA, PKC, PKG, Casein Kinase II (CK-II) and Golgi casein Kinase (G-CK). However, the only site that has been demonstrated to be involved in AQP2 trafficking is the PKA phosphorylation site (Kuwahara et al., 1995). A mutation of this site (S256A) or inhibition of PKA prevents the AQP2 shuttle, indicating that the phosphorylation of AQP2 by PKA is an essential step (Nishimoto et al., 1999; van Balkom et al., 2002a; Fushimi et al., 1997; Klussmann et al., 1999). A significant amount of the water channel

is constitutively phosphorylated and thus localized permanently in both the plasma membrane (van Balkom et al., 2002b).

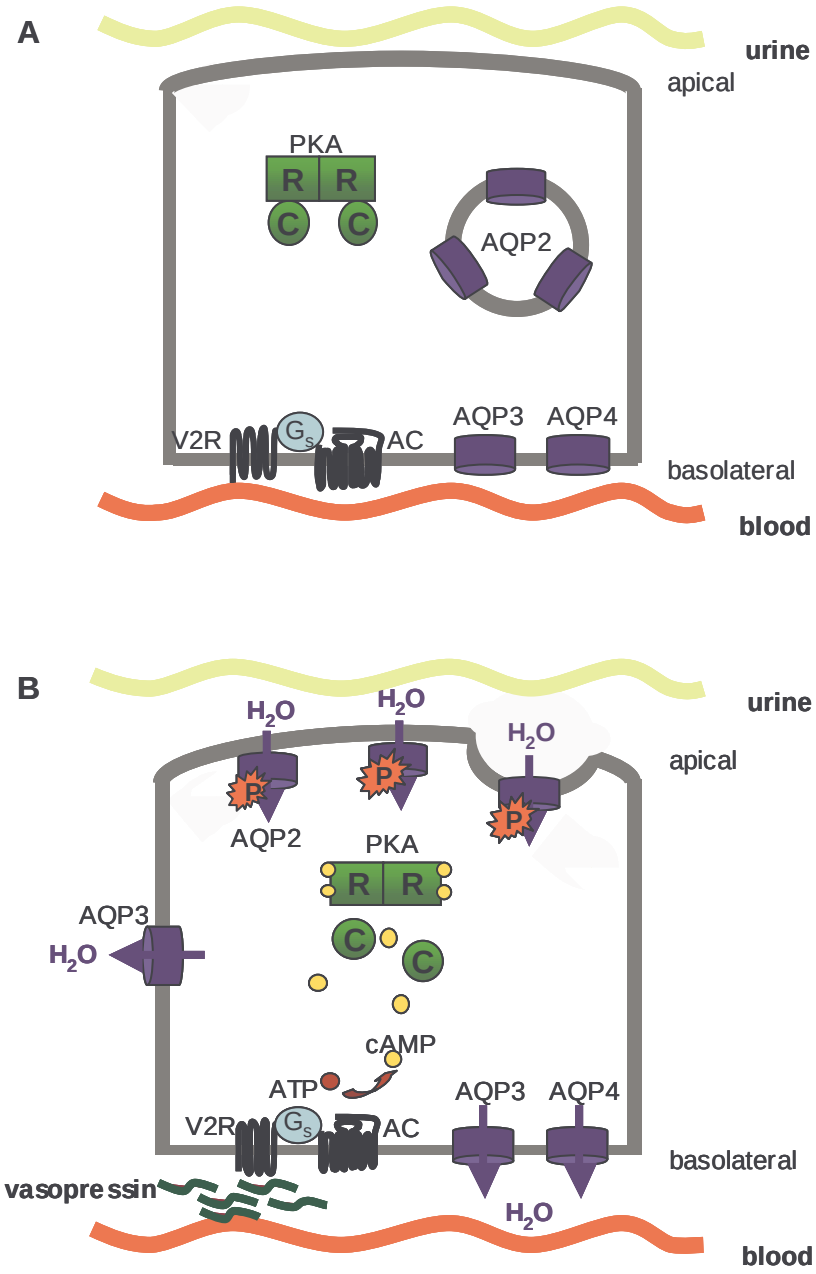

Figure 1.14 AVP-stimulated AQP2 redistribution In resting renal collecting duct principal cells, AQP2 resides on intracellular vesicles, **A**. After binding of AVP to the V2-receptor (V2R), adenylyl cyclase (AC) is activated *via* the G_s-proteins, and the intracellular cAMP concentration activates PKA. PKA phosphorylates AQP2 amongst other substrates. AQP2 is translocated to the plasma membrane and water enters the cell. Water exits the cell *via* AQP3 and 4 constitutively expressed in the plasma membrane, **B**.

1.7.3 AKAP18δ and AQP2 trafficking

In IMCD cells, AQP2 trafficking requires anchoring of PKA to AKAPs (Klussmann et al., 1999). Preincubation of cells with the synthetic peptide, S-Ht31, which prevents the binding between AKAPs and the regulatory subunit of PKA, significantly impairs hormone-dependent AQP2 trafficking. AKAP18δ co-localizes with AQP2 in IMCD cells and is present in both the soluble and particulate fractions derived from renal inner medullary tissue. Within the particulate fraction, AKAP18δ is identified on the same intracellular vesicles as AQP2 and PKA. AVP not only recruits AQP2, but also AKAP18δ to the plasma membrane. The elevation of cAMP causes the dissociation of AKAP18δ and PKA. The data suggest that AKAP18δ is involved in the AQP2 shuttle (Henn et al., 2004). AKAP18δ binds to PDE4D3 (Stefan et al., 2007; McSorley et al., 2006), thereby anchoring two important enzymes of the cAMP and vasopressin mediated water regulation to AQP2 bearing vesicles.

1.7.4 PDE4 and water reabsorption in the kidney

PDE4 plays an important role in the regulation of vasopressin induced AQP2 phosphorylation and the expression of AQP2 in the collecting duct. Mice with hereditary NDI show an increased activity of PDE3 and PDE4 in the kidney. Levels of cAMP in intact IMCD from NDI mice completely failed to increase in response to vasopressin. Treatment with the selective PDE4 inhibitor rolipram restored vasopressin-dependent cAMP accumulation in IMCD of NDI mice to the levels found in control mice (Homma et al., 1991). PDE3 and 4 inhibitor treatment significantly prevents the reduced expression of collecting duct AQPs and prevents the development of polyuria (Homma et al., 1991; Moses and Scheinman, 1993; Wang et al., 2002).

1.8 Aims

Heart contraction is hardly dependent on ß-adrenergic stimulation and following phosphorylation by PKA. In this work, AKAP expression and interaction partners in the heart are investigated.

Henn et al showed by a Northern blotting, that AKAP18δ mRNA is highly expressed in the heart (Henn et al., 2004; Trotter et al., 1999) This leads us to verify AKAP18δ expression on the protein level in cardiac myocytes.
We want to localize more precisely AKAP18δ expression inside the cardiac cell by microscopic and biochemical techniques, further we look for co-localization with known PKA target proteins. Depending on this co-localization, this work aims to find AKAP18δ interaction partners, and to define the function of the AKAP18δ complex in the PKA mediated signaling in the heart. The observation that AKAP18δ is found in SR-enriched fractions of cardiac myocytes and co-localizes with the SR proteins SERCA2a and PLB, led us to the hypothesis, that AKAP18δ is the AKAP involved in PLB phosphorylation.

Stefan et al showed a direct binding between AKAP18δ and PDE4D3 and identified potential binding sites. In co-transfected HEK293 cells. We want to verify and precise the parts of AKAP18δ involved in the binding.

PKA regulatory subunits exist in two different isoforms, named typeI and type II. PKA phosphorylation is shown to induce AQP2 translocation to the plasma membrane. In this work involvment of PKA-R typeI and/or typeII is investigated.