4 Discussion

4.1 Heart

Many AKAPs are known to be expressed in the heart (see table 1). Still some of the signals in RII-overlay detection of heart homogenate cannot be allocated to a known AKAP. Additionally, for a number of PKA targets the associated AKAPs are not identified. No AKAP is currently known to associate with the PKA substrates PLB or troponin I (Dodge-Kafka et al., 2006). AKAP18δ was first identified in rat renal inner medullary principal cells, but results from a Northern blot also show AKAP18δ mRNA expression in rat cardiac tissue. This led to the investigation of AKAP18δ protein expression in the rat heart. In this study, the presence of AKAP18δ in cardiac myocytes is demonstrated. Initially, AKAP18δ expression is detected in whole heart homogenates, and subsequently its intracellular localization to the SR is identified, where it directly interacts with PLB. This study also shows that AKAP18δ is involved in the regulation of Ca^{2+} re-uptake into the SR.

4.1.1 AKAP18δ is expressed in adult rat heart tissue

The presence of AKAP18δ in adult rat heart tissue is detected by various approaches: RII-overlay and Western blotting (Fig.3.1.1), or by immunoprecipitation (Fig 3.1.2 A), and by cAMP agarose precipitation and analysis of precipitates by RII-overlay and Western blotting (Fig.3.1.3). The observation of AKAP18δ in adult heart is also in agreement with the Northern blot analysis of the AKAP18δ expression pattern, showing that on the mRNA level AKAP18δ expression is strongest in the human heart (Henn et al., 2004).

The cAMP agarose precipitation of AKAP18δ and detection by Western blot shows its ability to bind regulatory PKA subunits in rat heart homogenates and confirms thereby that it functions as an AKAP in adult cardiac myocytes.

4.1.2 AKAP18δ is expressed in neonatal rat heart tissue

The neonatal cardiac heart shows different expression patterns for several proteins compared to the adult heart. The morphology and differentiation of the myocytes is developing during the first days of culture, contraction only starts 2-3 days after plating and differentiation into fibroblasts after one week. For Ca^{2+} measurements primary cultured neonatal cardiac myocytes are used, and AKAP18δ expression comparable to the adult heart is verified (Fig. 3.1.1 and Fig.3.1.2 C and D). AKAP18δ is detectable by RII overlay assay showing that AKAP18δ binds regulatory PKA subunits and functions as an AKAP in the neonatal heart. As in adult cardiac tissue, AKAP18δ is localized at the z-lines in neonatal cardiac myocytes (Fig. 3.1.6) (Lygren et al., 2007). These results show, that primary cardiac myocytes are a suitable model for studying the function of AKAP18δ.

4.1.3 Localization of AKAP18δ to the SR

The presence of a RII binding protein of 50 kDa in the heart fractions enriched for SR, and immunoblotting of the heart fractions revealed, that AKAP18δ is present in SR fractions together with PKA subunits (Fig. 3.1.4). The expression of AKAP18δ at the SR is confirmed by immunofluorescence microscopy showing co-localization of AKAP18δ with the sarcoplasmatic proteins SERCA2a and PLB at the z-lines of heart muscle in sections of adult rat heart tissue (Fig. 3.1.5) and by electron microscopy in primary cultured neonatal cardiac myocytes (Fig. 3.1.7). The presence of AKAP18δ in the SR fraction suggests that it has a role in targeting PKA to a protein localized in the SR and influencing its phosphorylation status (Lygren et al., 2007).

The mechanism by which AKAP18δ associates with the SR remains to be determined. AKAP18δ lacks the membrane targeting domain present in AKAP18α and AKAP18β (see Fig.1.15). Consistent with this finding, palmitoylation of AKAP18δ is not detected (Henn et al., 2004). Sequence analysis, however suggests that covalently bound myristoylation may mediate its attachment to a membranous compartment. Alternatively, AKAP18δ may

require a membrane-associated partner for the localization, as it is the case for AKAP18α which mediates the interaction with L-type Ca^{2+} channels through a leucine zipper-binding motif (Hulme et al., 2002; Burton et al., 1997).

4.1.4 PLB co-precipitates with AKAP18δ

Phospholamban is known to be phosphorylated by PKA, and co-localizes with AKAP18δ and PKA regulatory subunits in the heart SR (Figs. 3.1.4/5/7). PLB co-precipitates with AKAP18δ from ventricles of adult rat heart (Fig. 3.1.8) and from HEK293 cells co-transfected with PLB-YFP and AKAP18δ (Fig. 3.1.10, A) (Lygren et al., 2007). The interaction between PLB and SERCA2a is known to be direct and residues important for the interaction are mostly identified (Kimura et al., 1998; Toyoshima et al., 2003). HEK 293 cells do not express SERCA2a, indicating that co-precipitation of AKAP18δ and PLB is not due to interaction of both proteins with SERCA2a. Collectively, these experiments show, that AKAP18δ and PKA form a complex with PLB and SERCA2a *in situ* and AKAP18δ is associated to this complex by interacting with PLB.

4.1.5 PLB interacts directly with AKAP18δ

Co-precipitation studies are not sufficient to provide evidence for direct interaction between PLB and AKAP18δ. Therefore, peptide spot experiments are designed and direct binding between recombinant AKAP18δ-GST and synthetic PLB peptides fixed on a membrane is detected. In this experiments only the cytosolic part of PLB is sufficient for binding to AKAP18δ and the AKAP18δ core binding sequence in PLB is mapped to amino acids 13-21 (Fig. 3.1.9 A, underlined) (Lygren et al., 2007). The AKAP18δ core binding sequence of PLB is positioned at the end of domain IA (amino acids 1-16) and in the loop domain (amino acids 17-21) which is part of the hinge region between the two helical domains of PLB (Fig 1.11 A) (Metcalfe et al., 2004). This region is important for the inhibitory function of PLB for SERCA2a (Zamoon et al., 2005). The identified AKAP18δ binding region contains the PKA phosphorylation site (RRAS) for serine S16, as well as the Calmoduline kinase (CaMKII)-phosphorylation site T17. The cytosolic part of PLB is accessible for all cytosolic

proteins, and contains within 30 amino acid residues the interaction sites for SERCA2a, PKA, CaMKII, AKAP18δ and is accessible for dephosphorylation by protein phosphatase 1 (PP1). Presumably, not all proteins can bind at the same time because of steric hindrance. To sort the binding of so many proteins on the same binding region, there must be a mechanism regulating the binding, which lets to the investigation of the role of PKA phosphorylation in PLB for the AKAP18δ binding.

4.1.6 Influence of PLB Ser16 phosphorylation on the AKAP18δ binding

Phosphorylation often induces structural changes and influences binding or enzymatic activity of proteins. For example, PKA phosphorylation of PDE4D3 results in increased activity, altered rolipram sensitivity, altered Mg^{2+} sensitivity and re-programming of ERK phosphorylation (Houslay and Adams, 2003). Phosphorylation of PLB disrupts the functional interaction to SERCA2a (Asahi et al., 2000). After phosphorylation, PLB undergoes structural changes in the loop domain (residues 17-21) and the cytoplasmic helix (residues 13-16) is partially unwinding. Thereby PLB loses its secondary structure around the phosphorylation site, resulting in an unstructured region between the two α-helical loops with a concomitant loss of structural connections between the transmembrane and the cytoplasmic domains. Phosphorylation also increases the mobility of backbone motion across the entire protein (Metcalfe et al., 2005). These structural changes in PLB after phosphorylation could also influence the binding to AKAP18δ.

In the peptide spot experiment, introduction of the phosphorylated S16 abolished the binding to AKAP18δ-GST (Fig. 3.1.10). The results from the peptide spot experiment suggest that the AKAP18δ-PLB interaction is direct and possibly dynamically regulated by PKA phosphorylation of S16 providing an on/off mechanism. Experiments in neonatal myocytes with a short synthetic peptide of PLB cytosolic sequence with pS16 shows, that this peptide is not binding to AKAP18δ *in vivo* (Lygren et al., 2007).

Contradictory to the spot and peptide experiments, in co-transfected HEK 239 cells, PLB mutants mimicking the PKA phosphorylation by substitution of S16 by D or E, co-precipitate with overexpressed AKAP18δ (Fig. 3.1.11 A). Also *in vivo*, in adult rat heart homogenate, AKAP18δ can precipitate phosphorylated PLB (Fig. 3.1.13). It is to consider, that the synthesis of phosphorylated peptides is problematic, and cannot always be trusted.

An important difference between the peptide spotting and the immunoprecipitation experiment is, that on the membrane, PLB peptides are spotted as monomers, but in heart homogenate, PLB is also present in pentameric form. In this it is not clear, if *in vivo*, AKAP18δ binds pentameric or monomeric PLB. As well monomeric as pentameric PLB can be phosphorylated, and mutation studies suggest, that inhibitory action of PLB on the Ca^{2+} pump is exerted only by PLB monomers (Kimura et al., 1997). Also AKAP18δ is able to bind monomeric PLB peptides *in vivo* (Lygren et al., 2007). PLB pentamers possess phosphorylated and non-phosphorylated PLB monomers. A possible explanation for the divergence between the results of the peptide experiments and the immunoprecipitation experiment with whole heart homogenate is, that AKAP18δ does not bind phosphorylated PLB monomers, but is able to bind pentameric PLB through a non phosphorylated PLB momomer. After dissociation of PLB pentamers by boiling the samples in SDS, phosphorylated and non-phosphorylated monomers can be detected in the Western blot of the immunoprecipitates of AKAP18δ (Fig. 3.1.13).

Experiments on the intact heart show that ß-adrenergic stimulation induces phosphorylation of PLB at S16 by PKA and at T17 by CaMKII. Phosphorylation of both sites reverses the inhibition of SERCA2a by PLB. Phosphorylation of S16 of PLB is a prerequisite for the phosphorylation of T17 (Luo et al., 1998). The S16 site can be phosphorylated independently of T17 *in vivo* and phosphorylation of S16 is sufficient to evoke the maximal effect of ß-adrenergic stimulation (Chu et al., 2000). Mattiazzi et al. suggest that the T17 site of PLB has an important role, not only under physiological conditions such as ß-adrenoceptor stimulation, but also in the mechanical recovery under some pathological conditions such as acidosis and stunning (Mattiazzi et al., 2006).

Thus it would not be surprising, that PLB phosphorylation of T17 also would abolish binding to AKAP18δ.

4.1.7 HEK293 cells overexpressing PLB mutants and AKAP18δ-YFP

Immunoprecipitation experiments with co-expressed PLB deletion and substitution mutants and AKAP18δ in HEK293 cells do not confirm the results obtained by peptide spot experiments concerning the important residues for PLB-AKAP18δ interaction and the effect of PLB phosphorylation. PLB constructs lacking most of the cytosolic part co-precipitate overexpressed AKAP18δ, whereas constructs of only the cytosolic part of PLB do not co-precipitate with AKAP18δ. In the peptide spot experiments, only the cytosolic part of PLB is used and is sufficient for AKAP18δ-GST binding. Substitutions of R13, R14 and R25, or mimicking the PKA phosphorylation by substitution of S16 by D or E, do not inhibit co-precipitation with overexpressed AKAP18δ (Fig. 3.1.11, A), but inhibit binding in the peptide spot experiment. Possible reasons for the contradictory results obtained in the two experiments are that both techniques do not consider all components influencing binding *in vivo* in the heart and it is difficult to decide in which results to trust. On one hand, it is often problematic to spot synthetic peptides correctly, especially for phosphorylated peptides or hydrophobic amino acids. On the other hand, overexpressed membrane associated proteins, such as PLB or AKAP18δ could be misslocalized in HEK cells, not containing the proper intracellular structure for correct localization. Also the YFP tag could lead to improper targeting inside the cell. It would require an experimental setting using cells containing the same structural organized SR and cellular environment as in the living heart. Unfortunately, transfection rates of primary cultured cardiac myocytes yielded in our laboratory are not sufficient for biochemical experiments.

4.1.8 AKAP18γ is expressed in rat heart

Human AKAP18γ was cloned in 1999 by Trotter et al., it is a protein of 326 amino acids with a calculated molecular mass of 37 kD. The first 262 amino acids show 75% identity with AKAP18δ, the last 64 amino acids are identical to

all AKAP18 isoforms and include the conserved RII binding site (Trotter et al., 1999). Immunoprecipitates using an antibody recognizing all AKAP18 isoforms and the cAMP agarose precipitates show a weak signal at around 36 kDa in the RII-overlay, indicating that AKAP18γ is also expressed in the heart (Fig.3.1.2, A and 3.1.3, B). This confirms the results of Trotter et al., who found AKAP18γ RNA expression in the heart in a human multiple tissue Northern blot using a cDNA probe using the unique region of AKAP18 (nucleotides 357–689) (Trotter et al., 1999). In the immunofluorescence study, detection with the A18δ3 antibody, recognizing AKAP18δ and γ isoforms, yields a localization in striated pattern twice as narrow as using the AKAP18δ-specific antibody A18δ4 or α-actinin antibody. This indicates that AKAP18γ may localize in between z-lines, at the intramyofibrillar space or myofibrils sectioned at regions other than at the level of the z-disc. AKAP18δ differs from AKAP18γ in additional 33 amino-terminal residues, to which the antibody A18δ4 is directed (see Table 2 and Fig. 1.15). Immunoprecipitation with the A18δ4 antibody co-precipitates PLB, suggesting that the binding site of AKAP18δ for PLB is not located between residues 1-33, which are blocked by the antibody. Thus, we cannot exclude that in addition to AKAP18δ, also AKAP18γ is able to bind PLB. But the lack of an AKAP18γ specific antibody makes it impossible to prove or exclude the interaction between AKAP18γ and PLB. AKAP18α is expressed in the heart and interacts with the L-type calcium channels (Gray et al., 1998). Also binding of PLB to AKAP18α and β can not be excluded, as the C-terminus containing the RII binding domain is identical to all AKAP18 isoforms and specific antibodies for AKAP18α or β are not tested in co-immunoprecipitation experiments for PLB. Experiments to map the AKAP18δ binding site for PLB show, that probably residues between amino acid 124 and 220 of AKAP18δ are important for PLB binding (Lygren et al., 2007). This region is present also in AKAP18γ, but not in the isoforms α or β.

4.1.9 Influence of AKAP18δ on the Ca^{2+} re-uptake into the SR

Our data indicate that AKAP18δ recruits PKA to the complex containing PLB and SERCA2a. It may be important for regulating PKA phosphorylation of PLB at S16 and the PLB inhibitory effect on SERCA2a, thereby regulating the Ca^{2+}

re-uptake into the SR. AKAP18δ siRNA oligonucleotides increase the effect of NE, an agonist of the ß-adrenergic receptor, on the Ca^{2+} re-uptake into the SR, whereas control siRNA has no effect on NE stimulation (Fig. 3.1.14). This shows, that AKAP18δ is necessary to mediate ß-adrenergic stimulation in cardiac myocytes, presumably by anchoring PKA to PLB and thereby facilitating its phosphorylation (Lygren et al., 2007).

Upon chronic heart failure, adrenergic stimulation is a harmful compensatory mechanism in the failing human heart. β-Blockers give increased survival during post-infarction heart failure by preventing adrenergic stimulation (Miyata et al., 2000). Thus, targeting the SERCA2a/PLB/AKAP18δ/PKA complex to prevent increased Ca^{2+} re-uptake into the SR may be cardio-protective in post-infarction heart and presents an alternative to ß-blocker treatment. Blocking the binding between AKAP18δ and PLB may be achieved by applying direct antagonistic inhibitors which may be anchoring disruptor peptides or small molecular compounds binding to the PLB binding site of AKAP18δ and thus antagonize complex formation and signaling. A synthetic peptide of the cytosolic part of PLB succefully disrupts AKAP18δ/PLB binding *in vivo* by competitive binding. The peptide abolishes the striated distribution pattern of Akap18δ detected by immunofluorescence microscopy, indicating that the peptide disrupts the interaction of the two binding partners. The presence of the PLB peptide inhibits the increase in phosphorylation by almost 50%, indicating that Akap18δ is necessary for the recruitment of Pka to its target PLB (Lygren et al., 2007). This would prevent activation of SERCA2a in response to ß-adrenergic stimuli and hence prevent increased energy consumption believed to induce further damage leading to failure in the post-infarctic heart. Interfering with the cAMP pathway at other levels than ß-adrenergic stimulation provides a new concept for treatment of heart failure and may lead to a more effective therapy with less side-effects.

Chronic PLB-SERCA2a interaction is the critical Ca^{2+} cycling defect in dilated cardiomyophaty (CM) (Minamisawa et al., 1999). Targeting the SERCA2a activation by transfection of pseudophosphorylated PLB enhances myocardial SR Ca^{2+} re-uptake and promotes the improvement of progressive contractile dysfunction in a CM hamster model (Hoshijima et al., 2002). These findings

confirm the importance of PLB phosphorylation for the cardiac function, and indicate that targeting PLB phosphorylation is a powerful target for treatments against heart diseases.

4.2 Interaction of AKAP18δ and PDE4D

PDEs constitute the only cAMP-degrading mechanism in the cell and are expressed in the cardiomyocyte in at least 5 family variants, between them PDE4D (Mongillo et al., 2004). In AKAP18δ transfected HEK293 cells endogenous and transfected PDE4D co-immunoprecipitates with AKAP18δ , Fig. 3.2), and (Stefan et al., 2007). Direct binding is detected by peptide spotting experiments which indicate that four regions of AKAP18δ are involved in the binding (Stefan et al., 2007). Amino acids between the residues 201 and 301 of AKAP18δ are essential for PDE4D binding, as the AKAP18δ deletion mutant lacking the N-terminal residues up to residue 301 does not co-precipitate PDE4D (Fig. 3.2). This region of interest is identical in AKAP18δ and AKAP18γ, so binding of AKAP18γ to PDE4D cannot be excluded. Full length AKAP18δ co-immunoprecipitates PDE4D less efficient than the deletion mutants AKAP18δ-N67, AKAP18δ-N124, and AKAP18δ-N201, suggesting that the N-terminus negatively regulates the interaction with PDE4D.

Mongillo et al. (Mongillo et al., 2004) found in immunofluorescence studies, that PDE4D is expressed in cardiac myocytes in a striated pattern, overlapping with α-actinin, similar to the staining displayed for AKAP18δ (Fig. 3.1.5). Thus PDE4D and AKAP18δ are expressed in the same compartment, and may therefore interact in cardiac myocytes. PDE4D has an important role in the heart compartmentalizing cAMP after β_2-adrenergic stimulation, (Perry et al., 2002), e.g. by interactin with mAKAP on the RyR2 receptor (Fig.1.6) (Lehnart et al., 2005), having little or no effect on β_1-AR signaling (Xiang et al., 2005). PDE4D and mAKAP bound PKA co-localise at the RyR2 receptor as part of a negative feedback mechanism which may protect from excess β-adrenergic stimulation of Ca^{2+} transporters during cardiac excitation-contraction coupling. The interaction of PDE4D and AKAP18δ in cardiac myocytes would provide a negative feedback regulation for the PKA-PLB-SERCA2a complex. When cAMP levels rise after β_2-adrenergic stimulation, AKAP18δ bound PKA is activated. It activates and thus increases the activity of PDE4D bound in the complex. Subsequently cAMP levels decrease and PKA avtivity decreases. This may participate in the regulation of Ca^{2+} re-uptake into the SR after β_2-adrenergic stimulation. Further work will be necessary to determine whether a complex of SERCA2a/PLB/AKAP18δ/PKA/PDE4 exist in cardiac myocytes.

Specificity of cAMP signaling is achieved by tight control of the localization of the signaling events. Zaccolo and Pozzan 2002 showed that in rat cardiac myocytes, ß-adrenergic stimulation generates multiple microdomains of increased cAMP concentrations in the vicinity of the transverse tubule/junctional SR membrane with a diameter of approximately 1 µm (Zaccolo and Pozzan, 2002). These cAMP gradients activate a subset of PKA anchored to this region and diffusion of the second messenger is limited by the activity of phosphodiesterases. Impairment of cAMP-mediated signaling is often associated with heart failure (Movsesian, 1997) and several therapeutic approaches have been designed aiming at impaired cAMP concentration in cardiac myocytes, including PDE3 inhibition (Jaski et al., 1985; Movsesian, 1997). Selective inhibition of PDE3 is effective in the treatment of cardiovascular diseases by augmenting cardiac contractility and improving the clinical status in the short term but affect long term survival of patients in dilated cardiomyopathy (Packer et al., 1991; Movsesian, 1997). This let to the idea, formulated by R. Fischmeister 2006, that “good” and “bad” chronic effects of cAMP may reside in the capacity of the cell to maintain proper cAMP signaling microdomains. Proper cAMP signaling requires a strict localized control by AKAPs and PDEs, leading to modulation of only a limited number of substrates in a defined area. When compartments are disorganized, a global rise in cAMP and uncontrolled phosphorylation causes harmful diseases like cardiac hypertrophy and heart failure (Fischmeister et al., 2006). Thus treatment of heart failure by targeting AKAP anchoring of PKA should be a promising alternative to ß-blocker or PDE inhibitor treatment, targeting locally impaired cAMP concentrations.

4.3 Protein kinase A type II activation is sufficient to control the translocation of the water channel aquaporin-2 in the kidney

The presence of PKA on the same vesicles as AQP2 most likely facilitates AQP2 phosphorylation in response to AVP. Surprisingly, PKA types I and II are located in close proximity to AQP2 and only activation of PKA type II appears being necessary to induce the AQP2 shuttle (Fig 3.3.1). The finding that anchoring of PKA by AKAPs is necessary for the AQP2 shuttle (Klussmann et al., 1999) already suggests, that PKA type II must play an important role, as most AKAPs bind only PKA type II. Additionally, disruption of PKA anchoring on immunoisolated AQP2-bearing vesicles reduces vesicular PKA activity by about 50 % (Hundsrucker et al., 2006). However, it is unclear why both types of PKA are located in close proximity of AQP2. To test whether PKA type I contributes to the induction of the AQP2 shuttle, activators of PKA type I and inhibitors of PKA type II are not sufficiently selective to be used in immunofluorescence studies. Selective knock down of PKA type I or II by siRNA would be required to investigate the role of PKA type I.

Only a few AKAPs (dual specific AKAPs; D-AKAPs (Huang et al., 1997b)) can bind RI subunits and thereby anchor PKA type I to a specific compartment. The presence of PKA RI in the particulate (150,000 x *g* pellet) fraction and the presence of RI subunits on AQP2-bearing vesicles suggest, that D-AKAPs tether PKA type I to the vesicles. AKAP18γ, e.g., binds RI and RII subunits in oocytes (Brown et al., 2003) and is also expressed in the kidney (Trotter et al., 1999).

DbcAMP treatment did not affect the distribution of either RI or RII subunits but induced the AQP2 shuttle (see also Fig. 3.3.3). The observation that RI and RII subunits reside on AQP2-bearing vesicles but do not redistribute to the plasma membrane in response to cAMP elevation is consistent with previous findings showing that other proteins on AQP2-bearing vesicles including the motor protein dynein, the SNARE protein VAMP2 or the SNAP25-associated protein Hrs-2 do not shuttle with AQP2 (Gouraud et al., 2002; Marples et al., 1998; Tasken and Aandahl, 2004; Tasken and Aandahl, 2004). In contrast, AKAP18δ

resides on AQP2-bearing vesicles and translocates with AQP2 to the plasma membrane after elevation of cAMP (Henn et al., 2004). Elevation of cAMP causes the dissociation of RII subunits from AKAP18δ (Henn et al., 2004). This is in line with the observation that regulatory RII subunits of PKA do not redistribute to the plasma membrane in response to dbcAMP treatment. A similar mechanism may underlay the lack of the RI shuttle (see above).