Chapter 4

Discussion

4.1 AKAP7δ-derived peptides are potent disruptors of PKA-RII subunit anchoring

AKAP7δ is one of the AKAPs with highest affinity for binding of RII subunits of PKA [94]. The observation that truncated AKAP7δ binds RII subunits with higher affinity than the full length protein [71] led to the development of 25 amino acid residue long peptides derived from the RII-binding domain of AKAP7δ. The use of peptide substitution arrays, revealed that the peptides AKAP7δ-L304T-pep and AKAP7δ-L314E-pep possess a comparably high affinity for RII subunits as the wild-type (AKAP7δ-wt-pep) (Fig. 3.1). While the substitutions S305D and L308D abolished RII-binding. The peptides AKAP7δ-wt-pep, AKAP7δ-L314E-pep and AKAP7δ-L304T-pep effectively inhibit RIIα-binding to AKAP-derived RII-binding peptides (e.g. AKAP2, $AKAP_{IS}$ and Ht31) in RII overlay experiments.

Serine 305 of AKAP7δ is a predicted phosphorylation site for protein kinase C. Introduction of a negative charge by aspartate mimicking phosphorylation of this position, indicating that PKA-anchoring by AKAP7-isoforms

is putatively abrogated by PKC phosphorylation. As both proteins AKAP7 and PKC are stimulatory to calcium-signalling in cardiac myocytes [89, 127] this regulation may provide an interesting possible negative-feedback mechanism as part of heart contractile system. Recently, Bengrine *et al.* studied a feedback inhibition of the AKAP7α/β isoforms on the epithelial Na^+ channel [128]. Further, the authors demonstrated direct interaction between AKAP7α/β and PKC in soluble *Xenopus* oocyte fractions. Oocytes expressing AKAP7α/β exhibited a strong reduction in the amiloride-sensitive, whole-cell conductance (80% in high and 91% in low Na^+ conditions). The reduction of channel activity was PKA independent. These findings support the hypothesis that PKC may phosphorylate AKAP7-isoforms and thereby abrogate PKA anchoring, consistent with a PKA independent mechanism reported.

As the AKAP7δ-L308D-pep displayed abolished RII-binding but maintained helicity (see 3.2) it was utilised besides AKAP7δ-PP as additional negative control. The AKAP7δ-L308D-pep therefore serves as a new, probably more suitable type of negative control peptide as structural prerequisites for PKA binding are preserved.

Determining the binding affinity of the AKAP7δ-derived peptides to human RIIα subunits by SPR-measurements showed that AKAP7δ-wt-pep binds RIIα subunits with higher affinity ($K_d = 0.4 \pm 0.3\ nM$, see tabular 1.1 and 3.1) than Ht31 for at least one order of magnitude. The peptides AKAP7δ-wt-pep, AKAP7δ-L304T-pep and AKAP7δ-L314E-pep did not differ significantly in their absolute RIIα-binding affinity (Fig. 3.2, table 3.1). The SPR-measurements, however, revealed differences in RIIα-binding dynamics. The peptides differ in the velocity of the association and

dissociation phase, and show marginal dissociation compared to the most common utilised AKAP-PKA disruptor peptide Ht31 [93]. In experiments based on spot-synthesised peptides AKAP7δ-wt-pep, AKAP7δ-L304T-pep and AKAP7δ-L314E-pep display a considerable higher affinity for RIIα subunits than AKAP$_{IS}$ (Fig. 3.3).

A greater solubility in aqueous solution of AKAP7δ-L314E-pep and AKAP7δ-L304T-pep was observed compared to the peptide representing the wild-type sequence. AKAP7δ-wt-pep partially precipitated at a concentration of 30 μM limiting its experimental application. The peptide AKAP7δ-L314E-pep was still soluble at concentrations above 100 μM, indicating that the introduction of a glutamic acid residue increases its solubility. This explains the higher apparent RII-binding in the spot-based experiments compared to AKAP7δ-wt pep. Therefore AKAP7δ-L314E-pep is the most widely applicable peptide to study PKA-anchoring.

To test whether the AKAP7δ-derived peptides function as AKAP-PKA disruptors *in vivo*, two assays based on living cells were evaluated. AKAP7δ is one of several AKAPs tethering PKA to AQP2 (aquaporin-2)-bearing vesicles [72]. PKA activity was displaced from AQP2-bearing vesicles by the peptide AKAP7δ-L314E-pep (Fig. 3.4). Anchored PKA is necessary for the redistribution of AQP2 from the intracellular vesicles to the plasma membrane upon stimulation of the V_2 receptor by AVP in the principal cells of the kidney [98, 71]. Stearate-coupled and thus a plasma membrane permeable version of the peptide AKAP7δ-L314E-pep prevented the AVP dependent redistribution of AQP2 in IMCD cells (Fig. 3.6).

In cardiac myocytes, the tethering of PKA by AKAPs facilitates β-adrenergic receptor-mediated increases in L-type Ca^{2+} channels conductivity

and enhances contractility [89, 90]. The peptide AKAP7δ-L314E-pep prevented isoproterenol induced increases in L-type Ca^{2+} channel currents in neonatal cardiac myocytes (Fig. 3.5). The novel high-affinity AKAP7δ-derived peptides are thus valuable tools to investigate the role of PKA-anchoring for *in vitro* and *in vivo* studies.

4.2 Determinants of high affinity PKA-anchoring

In order to gain further insight into the interaction of PKA and AKAPs, the determinants defining the high-affinity interaction of the RII-binding domain of AKAP7δ with RIIα subunits were investigated. Previous studies had shown that the interaction between RII-binding domains and RII subunits involve hydrophobic amino acid residues of the amphipathic helix forming the RII-binding domain and that enhanced helix stabilisation does not directly translate into enhanced binding affinity for the RII-AKAP interaction [129, 94, 95]. Our studies confirm this observation for AKAP7δ. Furthermore, they indicate that additional factors safeguard the interaction and influence the binding affinity. Peptides comprising 25 amino acid residues derived from the RII-binding domain of AKAP7δ bind RIIα subunits with a subnanomolar K_d. Truncation mutants of less than 25 amino acids in length bind RIIα subunits less efficiently (Fig. 3.9). This observation cannot be explained by the loss of hydrophobic contacts as they were preserved in the truncation mutants. It is therefore likely that polar or charged amino acid residues contribute directly to the interaction, as their amounts were diminished in the truncation mutants. According to the structural model, eight

out of 13 polar or charged amino acid residues in the AKAP7δ RII-binding domain may form salt bridges or hydrogen bonds with partners in the binding pocket of the RIIα dimer (Fig. 3.8). Indeed, substitution of alanine for six of the potential salt bridge or hydrogen bond-forming amino acid residues decreased the binding affinity for RIIα subunits (Fig. 3.10), demonstrating their functional importance.

The argument is further supported by the finding that AKAP7δ-derived peptides of the same length substituted with oppositely charged amino acid residues bind RIIα subunits significantly less than wild-type peptides (e.g. D298R and E300R; Fig. 3.1). Furthermore, the substitution of alanine in position 311 for asparagine (N311A) led to a strong decrease in RIIα subunit binding (Fig. 3.10). This asparagine is conserved in the high-affinity AKAPs, AKAP7δ , AKAP2, AKAP5, AKAP12, and in AKAP$_{IS}$(Fig. 3.7). Despite the importance of the polar and charged residues, none of the tested alanine-substituted peptides lost the ability to bind to RIIα subunits (Fig. 3.10 B), suggesting that the interaction mediated by hydrophobic amino acid residues is sufficient for basal PKA binding.

Recently, Gold *et al.* published the NMR-structure of the peptide AKAP$_{IS}$ in complex with the dimerisation and docking domain of the RIIα subunit dimer [96]. The structural data supports the findings that hydrophobic contacts, intermolecular salt bridges and H-bonds contribute to AKAP-PKA binding. In particular, the amino acid residues N311 and K315 of AKAP7δ may be directly mapped to counterparts of the AKAP$_{IS}$ peptide (N15, G19, respectively) as H-bond partners of the RIIα subunit dimer. However, additional H-bonds or salt-bridge-forming amino acid residues of AKAP$_{IS}$ could not be directly mapped to the AKAP7δ. It appears that

AKAP7δ is slightly shifted in the binding groove formed by the RII dimer compared to AKAP$_{IS}$ and is capable of engaging in more putative salt bridge or H-bond formation. According to the authors [96], optimisation of AKAP$_{IS}$ for RIIα binding was in part achieved due to a double substitution putatively by introducing an additional H-bond. These data strongly corroborate the hypothesis that H-bond or salt-bridge-forming amino acid residues tighten AKAP-PKA binding.

Taken together strong evidence supports the enhancement of the model of AKAP-PKA interaction. As in addition to the well described amphipathic helix-forming RII-binding domain of AKAPs and hydrophobic amino acid residues in conserved positions, intermolecular hydrogen bonds and salt bridges contribute to the high-affinity binding to RIIα subunits.

4.3 Combination of bioinformatics and peptide spot-synthesis to identify new AKAPs

Utilising the newly gained information of the molecular determinants of AKAP-PKA interaction an algorithm for a database search for AKAPs was established. A pattern describing the observed amino acid residues in conserved positions was generated, representing the hydrophobic core contacts to the RII dimer. The charged amino acid residues serve as H-bond partners but may be delocalised within the peptide. Taking this into account, the P$_I$ range (3.0 - 6.4) derived from the RII-binding domains displayed in Fig. 3.7 was calculated as a measure of the delocalised charged amino acid residues. The database search was then restricted to sequences serving the pattern and

the calculated P_I range.

The such limited database search retrieved 4519 peptides further limited to 2572 peptides by a filter step (see 3.11, 3.12 and Appendix B). The filter step excluded duplicates but also reflected technical limits of the spot-synthesis. The general exclusion of cysteine-containing peptides (74.5% of all filtered peptides) might have missed putative AKAPs as for example the peptide derived from the previously described AKAP6 (KDAED<u>C</u>SVHNFVKEIIDMASTALKS) was excluded. As this was the major amount of peptides filtered (1381), these peptides might be tested for RII-binding in future by substituting cysteins to serines prior to spot-synthesis, which is assumed not to change RII-binding probabilities dramatically. Most of the other filtered sequences (15.0%) correspond to undesired amino acid accumulations in RII-binding domains (e.g. proline, or amino acid combinations that tend to be turn-building; compare 3.6). With these limits and the restriction to the human subset of expressed proteins a subset of previously described AKAPs was retrieved from the database (swissprot) and tested for RIIα-binding. In particular five RII-binding domains of the AKAPs displayed in Fig. 3.7 were not retrieved or excluded prior to spot-synthesis: AKAP6 (sequence contains a cysteine residue), AKAP13 (filtered as 'duplicate' as the sequence is identical with the control-peptide Ht31) and DAKAP550 (*Drosophila*-AKAP, not incorporated in the human database subset). The sequences of MAP2, AKAP3 and AKAP4 RII-binding domains were excluded from the pattern as they contain polar amino acids in the central conserved positions. The cognate AKAPs were thus excluded from the database search. Enhancing the pattern by these polar amino acids, while maintaining the pattern-symmetry leads to a very general pattern and by

this to too many hits for the following peptide spot-synthesis (approximately 12300 hits). This restriction may exclude several probably weak RII-binding AKAPs, but may strongly reduce the number of false hits as polar amino acids in these positions seem to be the exception. Additionally, AKAP4 was not incorporated in the human swissprot database at time of the sequences retrieval.

Amongst all peptides tested (including the RII-binding domains of the high affinity AKAPs AKAP2 and AKAP5) the AKAP7δ-derived peptides bound RIIα with the highest apparent affinity (see Fig. 3.12, Fig. 3.13 and Appendix B). All peptides that bound in the RII overlay assay (Fig. 3.12) were subjected to a competitive RII overlay assay to elucidate whether RIIα-binding proceeds in an AKAP like manner. The majority of peptides bound RIIα in the presence of the anchoring disruptor AKAP7δ-L314E-pep. One such peptide (RHG4) was utilised as positive control for the competitive RII overlay assay (Fig. 3.13). It is derived from the Rho GTPase-activating protein 4. Furthermore, the database search yielded 24 peptides derived from integrins, of which seven displayed RIIα-binding insensitive to AKAP7δ-L314E-pep. A peptide derived from α4 integrin precursor protein was also identified, however it did not display RIIα-binding. Lim *et al.* showed recently that α4 integrins bind to PKA type I but not to type II holoenzymes and that binding was insensitive to peptides disrupting AKAP-PKA interaction [130]. Similarly, Chaturvedi *et al.* demonstrated an interaction between ribosomal S6 kinase 1 (RSK1) and PKA of type I in complex, but not *via* D-AKAP1 [131]. Dependent on the activation state of RSK1, the kinase influences the activity of PKA and the ability of cAMP to stimulate PKA. The authors report, that inactive RSK1 interacts with RI subunits and active

RSK1 interacts with the C subunits. These findings were recently embedded into a wider context [132]. Thus, the proteins whose peptides bound to RIIα in presence of AKAP7δ-L314E-pep may – at least in part – bind to PKA by a novel mechanism. This however, remains to be demonstrated.

Peptides that displayed RIIα-binding abolished upon treatment with the anchoring inhibitor peptide AKAP7δ-L314E-pep were treated as putative RII-binding domains (Fig. 3.13). This resulted in 14 peptides displaying weak but stable RIIα-binding with cognate proteins not described as AKAPs (Fig. 3.13; swissprot identifiers: ATY3, CN129, FAK1, HIP1, IKAP, IP3T, K13B, K406, LR1B, MRIP, SYM, SYTC, TLN2, TMS3 for protein description see Appendix B). The weak binding may be the reason that these proteins were not detected as AKAPs before. The identified peptides represent putative new RII-binding domains. The CN129 protein was chosen to be further tested for AKAP function.

4.4 Characterisation of the AKAP function of the CN129 protein

The CN129 protein was identified *via* the database search as a new AKAP by its putative RII-binding domain. An array of overlapping peptides for the full length CN129 and a substitution array of the CN129 RII-binding peptide revealed that the previously identified peptide is indeed the one with the highest apparent binding affinity for RIIα subunits within the protein (Fig. 3.14). The CN129-peptide also displays the 'typical' properties of a RII-binding domain: reduced or abolished binding to RIIα subunits by introduction of proline in the core binding domain or by substituting the

hydrophobic amino acid residues in conserved positions by charged or polar amino acid residues (Fig. 3.15). Additional to the thus characterised RII-binding domain, several of the overlapping peptides displayed RIIα-binding. Non-canonical RIIα-binding was not observed for the CN129 protein as the partial CN129-CFP fusion protein, containing prolines in the RII-binding domain displayed no RIIα-binding (Fig. 3.16). Taking the structural properties of CN129 into account (Fig. 3.19), only the C-terminal, α-helical region might serve as an additional canonical RII-binding domain as it displays an amphipathic character. The partial CN129-CFP fusion proteins lacking the C-terminus did not show reduced apparent binding (Fig. 3.16). Thus, evidence for this putative second RII-binding domain remains to be provided.

To test whether the CN129 protein functions as AKAP, human and rat partial (amino acids 1-125), full length CN129-CFP fusion proteins and proline containing versions were generated. These constructs were overexpressed in HEK293 cells and tested for RIIα-binding in RII overlay assays. The RII overlay assay indicated clearly that the CN129 protein functions as AKAP as signals at the appropriate size were detected in the autoradiography and abolished upon treatment with the anchoring disrupting peptide AKAP7δ-L314E and for the double proline point-mutated constructs (Fig. 3.16). Compared to AKAP7δ, CN129 displays weak RII-binding as expected from the spot-synthesised peptides. The double band detected in protein preparations for cells expressing CN129-CFP fusion proteins may represent CN129 and additionally a degeneration product, a posttranslational modification or another AKAP with similar size bound to CN129. The upper band appears to shift for the partial CN129-CFP constructs. Thus, also dimerisation combined with posttranslational modification of one of the monomers may be assumed. De-

duce further that posttranslational modification of CN129 may be influenced by N-terminal α-helix.

To gain insight into the subcellular distribution as a hint to the targeting of CN129, the human full length CN129-CFP was overexpressed in several cell lines (SH-SY5Y, WT10, Cos7 and HEK293) and analysed by laser scanning microscopy. Cytosolic and nuclear distribution of CN129-CFP was observed for all cell lines tested, but was excluded from unidentified subcellular regions (Fig. 3.17). This suggested a targeting for CN129 with a yet unknown underlying mechanism. However, CFP might mask the proper subcellular distribution of CN129 as the CFP protein is approximately twice the size of CN129. Thus, the actual subcellular distribution of CN129-CFP, a comparison of the distributions of endogenous, overexpressed untagged and the CFP fusion proteins by use of a CN129 specific antibody is necessary.

The high conservation of CN129 within vertebrates implies an important role in cellular function. RIIα subunits of human and zebrafish (*Brachydanio rerio*) share a 59% amino acid identity in the dimerisation and docking domain, a RIIα subunit for *Tetraodon nigroviridis* is not described. The peptide derived from the putative RII binding domain of the fish orthologue of *Tetraodon nigroviridis* differs from that of zebrafish in two amino acid residues and binds to human RIIα subunits, indicating an obviously conserved AKAP function. As almost the complete CN129 protein is conserved, additional putative scaffolding function for CN129 could be expected. The CN129 protein is the first AKAP whose NMR-structure is solved (Fig. 3.19). The structure of CN129 is highly ordered, with the amphipathic α1-helix forming the RII-binding domain and the C-terminally exposed α3-helix suggesting a putative protein-protein interaction site. The structural prop-

erties however lead to the problem, that only a major conformational change allows RII subunits to access the RII-binding domain. A task that might be clarified in future by solving the structure of AKAP CN129 in complex with RII subunits.