# Chapter 1

# Introduction

Cells receive environmental information directly from neighbouring or far distant cells and tissues by local or systemic mediators such as nitric oxide, neurotransmitters or hormones. One of the underlying cellular mechanisms processing only relevant information is a set of membrane-bound receptors. These receptors transduce upon binding of distinct mediators, the receptor ligands, a specific signal through second messengers into the inner cell. Second messengers are ubiquitously distributed and the same second messenger may be generated simultaneously to different receptor simulations within the same cell.

This raises the question how the specific signal may be transduced specifically. Interactions between proteins are one prerequisite for the specific signal transduction on the subcellular level. The cellular response to a number of hormones is characterised by the activation of signalling cascades which change the state of phosphorylation of various proteins [1, 2]. The phosphorylation of proteins is a reversible process that is mediated by two classes of enzymes: protein kinases which catalyse the phosphorylation and protein phosphatases which catalyse the reaction of dephosphorylation [2]. The ac-

tivity of kinases and phosphatases is regulated by second messengers as $Ca^{2+}$, phospholipids, cyclic guanosine monophosphate (cGMP) or cyclic adenosine monophosphate (cAMP). Numerous protein kinases and second messengers appear in various organisms and cell types. Protein kinases such as protein kinase A (PKA) catalyse the transfer of the terminal phosphate group from adenosine triphosphate (ATP) to serine, threonine or tyrosine residues in consensus sequences of proteins. One possibility to rise the specificity of the cellular response to a certain signalling molecule is spatial restriction, or compartmentalisation of the enzymes through association of the enzymes to anchoring proteins which localise them close to their regulators and substrates. Anchoring proteins that possess the ability to anchor several enzymes at the same time are termed 'scaffolding proteins'. These scaffolding proteins serve as focal points of integration for different signalling pathways and for the precise mediation of a signal within a signalling cascade [3].

## 1.1 Signalling of cyclic adenosine monophosphate

Among the receptors located at the plasma membrane, the G-protein coupled receptors (GPCRs) form the majority comprising a super-family with more than 1000 members. They are characterised by conserved seven transmembrane helices and coupling to guanine nucleotide binding proteins (G-proteins) which mediate signalling in response to activation of the receptors. Ligands of the GPCRs are for example peptide hormones such as arginine-vasopressin (AVP) for the Vasopressin 2 receptor (V2R) or adrenaline for the $\beta$-adrenergic receptor [4, 5]. Activated GPCRs release the bound het-

erotrimeric G-proteins mediating the signalling by their action on downstream substrates such as adenylyl cyclases [6, 7, 8]. G-proteins are named after their GTPase activity converting bound guanine nucleotide triphosphate (GTP, active state) slowly to guanine nucleotide diphosphate (GDP, inactive state). They are divided into small G-proteins and heterotrimeric G-proteins. Only the latter are coupled to GPCRs. They consist of three subunits ($\alpha$, $\beta$, $\gamma$), that are further subdivided. Upon receptor stimulation the $\alpha$ subunit dissociates and – in the case of $G_s$ – stimulates adenylyl cyclases (ACs). The released $\beta\gamma$ subunits regulate the activity of a variety of signalling molecules such as phosphatidylinositol kinases, phospholipases, receptor kinases and also adenylyl cyclases [9, 10, 11].

The adenylyl cyclases family consists of ten members of adenylyl cyclases (ACs) numbered AC1 to AC9 and the sperm-specific sAC [10]. Adenylyl cyclases catalyse the conversion of adenosine triphosphate (ATP) to cyclic $3'$-$5'$ adenosine monophosphate (cAMP). In addition to the regulation by G-proteins, they are synergistically regulated by $Ca^{2+}$/Calmodulin (AC I, III and VIII) or inhibited by PKA or PKC (AC V and VI). The ACs are localised mainly in micro-domains of the plasma membrane, the so called lipid rafts. As ACs provide the starting point of cAMP signalling by generating the second messenger, they are also part of the mechanisms underlying the termination of cAMP signalling. By inhibition of the AC or by abortion of G-protein mediated stimulation (return to the GDP-bound state) the AC stops cAMP production.

Key players in the termination of cAMP signalling are cyclic nucleotide phosphodiesterases (PDEs) as they hydrolyse cAMP and cGMP. The superfamily of PDEs comprises more than 30 PDEs which are sub-grouped into

eleven different families [12]. Among these PDEs the families PDE4, PDE7 and PDE8 specifically hydrolyse cAMP. The propagation of the cAMP signalling is provided by cAMP binding to cyclic nucleotide gated channels (cNGC) to modify their open probability, to 'Exchange proteins directly activated by cAMP' (EPACs) influencing cell morphology or to its major target, PKA.

## 1.2 Protein kinase A (PKA)

The PKA substrates are distributed throughout the whole cell [13]. PKA forms a tetrameric holoenzyme in its inactive state. Two catalytic subunits (C) are inhibited by association with regulatory subunits (R) that form dimers. The catalytic subunit isoforms C$\alpha$, C$\beta$ and C$\gamma$ are encoded by three genes within *mammalia*. The regulatory subunits RI$\alpha$, RI$\beta$, RII$\alpha$ and RII$\beta$ are encoded by four genes [14, 15, 16, 17, 18, 19, 20]. The PKA holoenzyme is named after the type of regulatory subunits present: Type I if RI$\alpha$ or RI$\beta$ are present, type II if RII$\alpha$ or RII$\beta$ are present. The PKA of type is mainly localised in the cytoplasm [21, 22]. The ubiquitously expressed RI$\alpha$ subunit exists in two splice variants [23]. Expression studies revealed, that the transcript 1b of RI$\alpha$ is more abundant in adrenal tissue than the transcript 1a [24]. The RI$\beta$ subunit is mainly expressed in brain tissue. Up to 75% of PKA type II is anchored to subcellular structures [21, 22]. RII$\alpha$ is expressed ubiquitously. RII$\beta$ is mainly found in brain, neuroendocrine and adipose tissue, in the granulosa cells of the ovary and in the sertoli cells of the testis [19]. The catalytic subunits display similar kinetics and specificity to their substrates [25, 26, 27]. The activation of PKA is mediated by the binding of two molecules cAMP to each regulatory subunit. This lowers the

binding affinity of the dimer of R subunits to the C subunits [26, 28], the latter are released and phosphorylate their substrates at serine or threonine residues which are embedded in the consensus motif $[RK](2)-X-[ST]-X$ [29, 30, 31, 32].

Animal models (homo or heterozygous knockout mice) for all but the PKA C$\gamma$ subunit exist and display a range of phenotypes form healthy appearing mice for RII$\beta$ knockouts to early postnatal lethality for a approximately 73% of the C$\alpha$ knockout (KO) mice. Compensatory increase in levels of the remaining PKA subunits was observed in KO animals. Phenotypes of the C$\alpha$ knockouts comprise embryonic lethality, runted mice with deficits in liver function, sperm motility and diminished production of the major urinary proteins in kidney. C$\beta$ knockout mice showed a defect in hippocampal synaptic plasticity including long-term depression and -potentiation. RI$\alpha$ KO mice displayed similar phenotypes as patients suffering from Carney complex (carrying by 65% mutations in the RI$\alpha$ gene, resulting in haploinsufficiency), including altered heart rate variability and propensity for extracardiac tumorigenesis. Further, embryonic fibroblasts showed constitutive PKA activation and immortalisation due to upregulation of D-type cyclins. Mice lacking the RI$\beta$ subunit of PKA are defective for hippocampal synaptic plasticity including long-term depression and -potentiation. The latter phenotype is also observed for RII$\alpha$ KO mice in and in addition ocular dominance plasticity (ODP) in the developing visual cortex. RII$\beta$ KO mice showed protection against diet-induced obesity, insulin resistance, dyslipidemia and fatty livers [33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49].

## 1.3 A kinase anchoring proteins (AKAPs)

A kinase anchoring proteins (AKAPs) encompass a family of proteins defined by their ability to bind regulatory subunits of PKA. The first AKAPs, MAP2 and AKAP75, were discovered in preparations of the PKA holoenzyme of type II [50, 51]. Since then more than 50 members of the AKAP protein family have been identified [52]. In an average cell 5-10 different AKAPs are expressed [53]. AKAPs show poor sequence homology and differ in regulatory subunit binding affinity. They are functionally conserved by their interaction with regulatory subunits of PKA holoenzyme of type I or type II. A characteristic of AKAPs is their ability to bind PKA and anchor it to subcellular compartments. Most AKAPs bind regulatory RII$\alpha$ and RII$\beta$ subunits. On the other hand so called dual specificity AKAPs (D-AKAP) bind to RII and regulatory subunits of type I [54, 55, 56, 57]. The only known AKAP that binds exclusively to RI subunits is AKAP$_{CE}$ in *Caenorhabditis elegans* [58, 59]. The properties of AKAPs are summarised in Fig. 1.1. Historically, AKAPs were named after a former introduced name (e.g. Map2) or according to their apparent molecular weight, observed like 'AKAP15/18', however multiple splice variants that differ in molecular weight were discovered amongst the AKAPs, for example AKAP18$\alpha$ (9.0 kDa), $\beta$ (11.5 kDa), $\gamma$ (37.1 kDa) and $\delta$ (39.5 kDa). The Gene Nomenclature Committee renamed the AKAPs by running numbers AKAP1 - 13, however maintained former names like Map2 (reviewed in [60]). This new nomenclature was adapted throughout this work.

For the AKAPs AKAP1, AKAP4, Map2, Ezrin and WAVE-1 KO mice were generated displaying severe phenotypes. AKAP1 KO females are subfertile (approximately a third of the number of litters compared to wild-

type), AKAP4 KO male mice are infertile (lack of sperm motility), Map2 KO showed no apparent abnormalities, Ezrin KO did not survive for longer than 1.5 days post-natally. The early postnatal mice displayed substantial retardation in the development of photoreceptors. About a third of WAVE-1 KO progeny died within 24-48 h of birth. For the surviving mice, the brain-specific AKAP WAVE-1, a member of the Wiskott-Aldrich syndrome protein (WASP) family, showed a reduced size and behavioural abnormalities (deficits in sensorimotor function and cognition). The severe phenotypes of the AKAP KO mice indicate the importance of proper PKA-anchoring [61, 62, 63, 64, 65, 66, 67, 68].

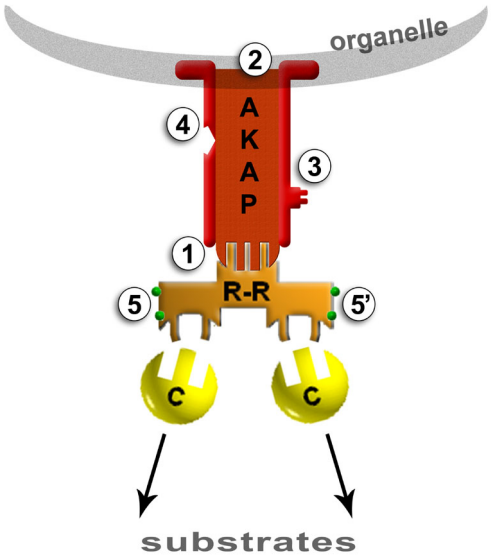


Figure 1.1: Model of an AKAP. Schematically displayed are the properties of AKAPs. The anchoring or RII-binding domain (1), the targeting domain (2), optionally additional protein-protein interaction domains (3), and a catalytic core of an AKAP are indicated. Upon stimulation by cAMP (5, green globes) the regulatory dimer (R-R) of PKA releases the catalytic subunits (C) which in turn phosphorylate their substrates.

### 1.3.1 Subcellular targeting of PKA by AKAPs

AKAPs target PKA to a wide range, including nuclear envelope, Golgi apparatus, mitochondria, cytoskeleton and plasma membrane. The targeting domains may be protein-protein interaction sites or domains that bind to phospholipids or may result from post translational modifications such as prenylation. On one hand several AKAPs may bind to the same compartment, on the other splice variants derived from one AKAP gene can target to different compartments. Examples for two different AKAPs that target to the same compartment are AKAP5 and the $\alpha$- and $\beta$- isoforms of AKAP7. However the underlying mechanism for targeting the same compartment are different: AKAP5 binds phospholipids through a N-terminal peptide sequence whereas the AKAP7 isoforms are recruited to the plasma membrane *via* myristoyl and dual palmitoyl groups [69]. The AKAP7$\gamma$ and $\delta$ isoforms are located mainly in the cytosol due to the lack of the targeting domain present in $\alpha$ and $\beta$ (see also Fig. 1.2, [70, 71]). For AKAP7$\delta$ PKA-anchoring to AQP2-bearing vesicles is observed [72]. Targeting of AKAPs to different subcellular compartments is summarised schematically in Fig. 1.2.

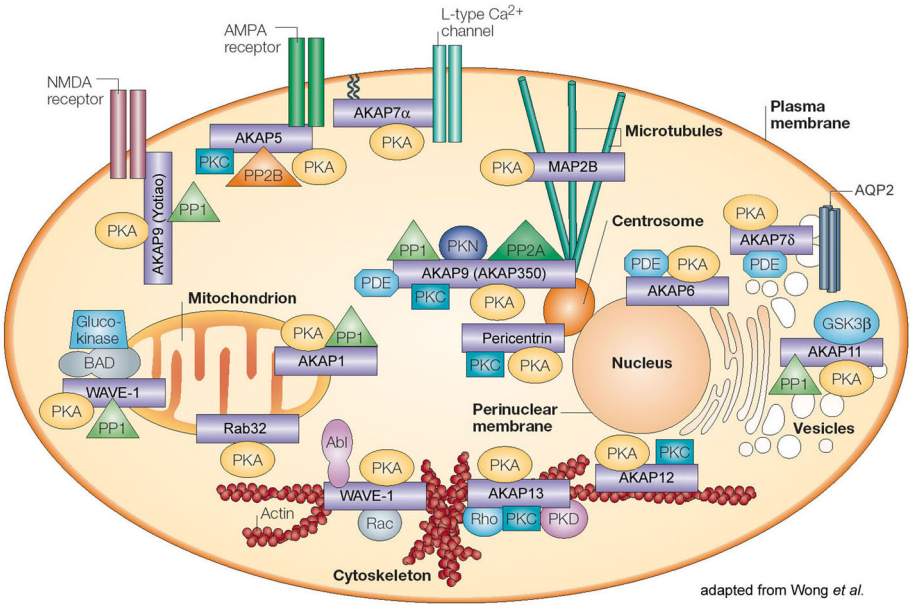


Figure 1.2: AKAPs target PKA to specific subcellular compartments, including the plasma membrane, mitochondria, cytoskeleton, centrosome and nuclear envelope. By recruiting additional enzymes, AKAPs create focal points for signal transduction. As exemplified by AKAP7$\alpha$ and AKAP7$\delta$, splice variants of one AKAP may display different targeting (L-type $Ca^{2+}$ channels or AQP2-bearing vesicles) and recruit different subsets of enzymes. For an elaborate description see Wong and Scott, 2004 [69])

### 1.3.2 Interaction of AKAPs and PKA type II

The binding between AKAP and PKA is mediated by the so called anchoring domain or RII-binding domain of an AKAP that binds to the dimer of regulatory subunits of the PKA. The RII-binding domain of the AKAP forms a conserved structural motif: an amphipathic $\alpha$-helix. This structural element was initially predicted from a fragment of an AKAP-derived from human thyroid, AKAP Ht31 [73], and confirmed by nuclear magnetic resonance (NMR) studies for a peptide derived from the RII-binding domain of Ht31 (the peptide Ht31) and for a peptide derived from the RII-binding domain of AKAP5 [74]. Investigating helical wheel projections in the area of the RII-binding domain for more than 20 AKAPs, the distribution of hydrophobic

or hydrophilic amino acids on the opposing faces of the helix was deduced [75, 76]. AKAPs are characterised by binding to regulatory subunits mediated by such an amphipathic helix (see below). An exception of this rule is the AKAP Pericentrin, which binds to regulatory subunits but does not display an amphipathic helix [77]. A multiple sequence alignment of 15 different AKAPs led to a common RII-binding motif: X-($\phi$)-X-X-X-(A,S)-X-X-($\phi$2)-X-X-($\phi$2)-X-X-(A,S)-($\phi$). X indicates any amino acid, $\phi$ amino acids valine, leucine or isoleucine, A alanine and S the amino acid serine [76]. The amino acid proline prevents the formation of an $\alpha$-helical conformation whereas there is only minor influence on the amphipathic nature of the helix [73]. The introduction of proline at different sites of the RII-binding domain of Ht31 prevents the binding to the RII subunit of the PKA, indicating that the amphipathic helix is required for RII-binding. Other mutagenesis studies highlighted the influence of hydrophobic amino acids with long aliphatic side-chains (valine, leucine, isoleucine) [78]. NMR studies displayed the structural complex of the N-terminal RII$\alpha$ (amino acids 1-44) dimers in association with the RII-binding domain of Ht31 or AKAP5, respectively. These data point out that direct helix-helix contacts of the hydrophobic amino acids mediate the contact between AKAP and PKA [74]. It took several experimental steps to identify the responsible amino acids for dimerisation and AKAP binding of the RII subunits. These are the first 30 N-terminal amino acids, where deletion of the first 5 amino acids lowers the binding of AKAPs remarkably whereas the dimerisation of the RII subunits is still maintained. Within these first 5 amino acids the isoleucines in position 3 and 5 determine the association with AKAPs [79, 80, 81, 82]. The three dimensional structure of the N-terminus (amino acids 1-44) of the regulatory subunit RII$\alpha$ was deter-

mined by NMR [83, 84, 74]. Two of the RII$\alpha$ termini form a dense packed anti-parallel dimeric four helix bundle of the X-type. Phosphorylation of a threonine residue in position 54 of the peptide sequence of RII$\alpha$ subunits led to a modulation of the binding to AKAP8 and AKAP9 [85, 86] and thereby regulates the binding between AKAP and RII subunits.

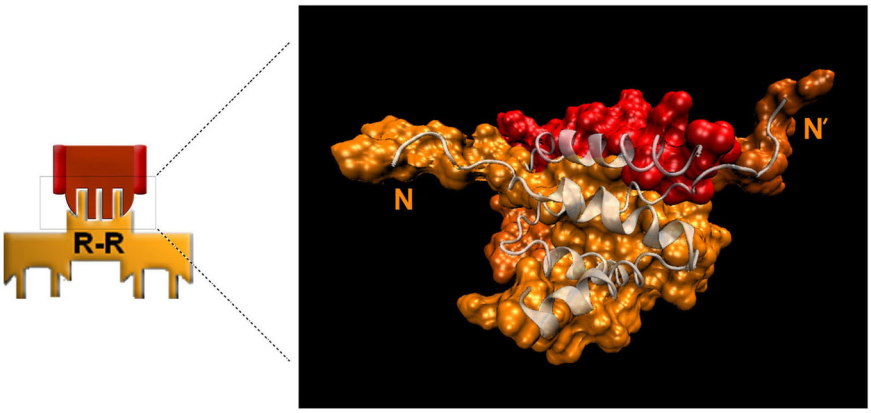


Figure 1.3: Molecular basis of the AKAP-PKA interaction. Depicted is the interaction of the AKAP RII-binding domain (red) with the dimerisation and docking domain of the RII$\alpha$ subunit (orange, amino acids 1-44) schematically and as molecular structure (based on the pdb-entry 2DRN, see text). Merged surface and ribbon representations indicate the helical contacts of the AKAP peptide (top, red) to the X-type four helix bundle of the RII dimer. RII protomers are displayed by light and dark orange, their N-termini are distinguished by apostrophes.

### 1.3.3 AKAPs as scaffolding proteins

Compartmentalisation involves scaffolding proteins concentrating signalling molecules at specific sites within cells, thereby limiting their access to only a subset of their targets. AKAPs are prototypic scaffolding proteins [60, 69]. For example, AKAP7$\delta$ binds PKA and PDE4D3/9 at AQP2-bearing vesicles [72] establishing a negative feedback loop in cAMP signalling dynamics. Thus this scaffold is capable of the micro-environmental regulation of cAMP lev-

els. An example for AKAP7$\alpha$-anchored PKA signalling modulating L-type $Ca^{2+}$ channel currents is depicted in Fig. 1.4. The scaffolding properties of AKAP5 (human or rat orthologue AKAP79 or AKAP150, respectively) were dissected by the usage of RNAi [87]. AKAP5 constructs lacking the binding domains for PKC, protein phosphatase 2 B (PP2B) or PKA in the human (AKAP79) and rat (AKAP150) orthologue served for a series of experiments. The endogenous AKAP5 of either human or rat-derived cells was silenced by RNAi and cells were reconstituted with the modified corresponding orthologues. This approach showed that AKAP79/150 coordinates different enzyme combinations to modulate the activity of two neuronal ion channels, the AMPA-type glutamate receptors and the M-type potassium channels (see also Fig. 1.2). For functional signalling the first requires anchored PP2B, synapse-associated protein 97 and PKA, whereas the latter only requires anchored PKC. This work points out the concept that AKAPs are integrating points and modulators for signalling, specifying or diversifying signalling dependent of the contextual enzyme combination engaged or active.

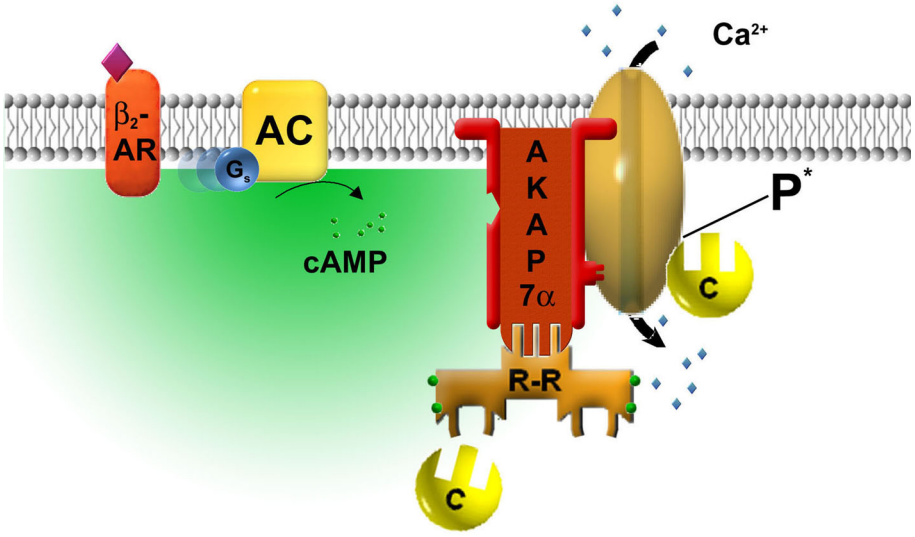


Figure 1.4: Involvement of AKAP7$\alpha$ in the regulation of the L-type calcium channel. In cardiac myocytes, L-type $Ca^{2+}$ channels are phosphorylated by PKA in response to $\beta$-adrenergic receptor activation [88, 89, 90]. Phosphorylation increases the open-probability of the channels and is a key event in $\beta$-adrenergic-receptor-mediated increases in myocyte contractility. Upon stimulation of the $\beta_2$-adrenergic receptor, the coupled G-protein subunit $G_{\alpha s}$ dissociates and activates adenylyl cyclase leading to production of cAMP. cAMP activates PKA anchored by AKAP7$\alpha$ *via* a leucine-zipper motif to L-type $Ca^{2+}$ channels. PKA catalytic subunits dissociate and phosphorylate, enhancing thereby the open probability of the L-type $Ca^{2+}$ channel leading to an increase of cytosolic $Ca^{2+}$-levels.

## 1.4 Peptides as disruptors of PKA-anchoring

Utilising synthetic peptides as competitive disruptors of protein-protein interactions allows to gain insight into the function of selected protein-protein interactions. Generating peptides derived from native proteins that mimic a known (or assumed) binding domain possess *a priori* specificity for at least a family of protein-protein interactions. For the successful generation of such peptides certain prerequisites concerning the topology (linear) and size ($\leq$ 27 amino acids) of the interacting surface/domain, structural flexibility, sequence composition (see 2.3) and the physical and chemical accessibilities must be fulfilled by the peptide meant to be generated. Recent studies took advantage of the use of so called 'spot-synthesised'peptide. Spot-synthesised peptides consist of up to approximately 27 amino acids in length, which are synthesised on cellulose membranes. Such membranes or 'peptide arrays'are utilised to elucidate the influence of single amino acid to binding. This technique allows the optimisation of peptides for binding or for proteome wide studies of protein-protein interaction. Peptides can be easily modified for visualisation, immobilisation or cell permeation by fluorescent dyes, affinity tags, or by addition of cell penetrating tags or sequences (e.g. stearate, poly-arginine, Penetratin, MAP- or Tat-peptide; reviewed in [91, 92]). The possibility to utilise DNA-vectors which encode peptides (potentially as GFP-fusion proteins) and transfect cells with these constructs opens the technical field to manipulations by molecular biology in addition to pharmacological administration of synthesised peptides. Thus, taking advantage of assays based on living cells and thereby even to overcome chemical synthesis problems (see 2.3 and Discussion).

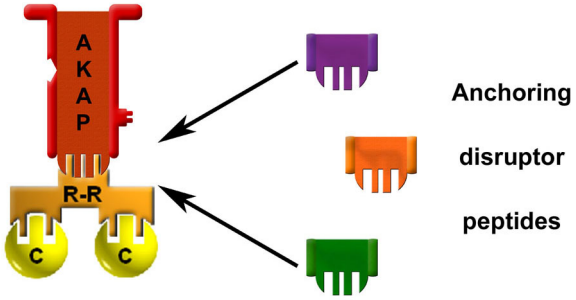


Figure 1.5: Peptidic disruptors of the AKAP-PKA interaction mimic the RII-binding domain of AKAPs.

As disruptors of the AKAP-PKA interaction, several peptides were generated. Such peptides mimic the anchoring domain of AKAPs (Fig. 1.5). The first peptide generated was Ht31 derived from the RII-binding domain of AKAP13 (Ht31/AKAP Lbc, [93]). The equilibrium dissociation constant ($K_d$) for the interaction of the peptide Ht31 with RII$\alpha$ subunits is $4.0 \pm 1.2\ nM$. It was determined by equilibrium dialysis. In later studies, the affinity of the peptide Ht31 to RII$\alpha$ and RI$\alpha$ subunits was determined by fluorescence polarisation measurements (RII$\alpha$: $K_d = 2.2 \pm 0.03\ nM$; RI$\alpha$: $K_d = 1.3 \pm 0.06\ \mu M$ [94]). *In vitro* analyses and cellular assays revealed that the peptide Ht31 functions as a non-selective disruptor of AKAP-RI and AKAP-RII interactions. Alto *et al.* [94] determined the minimal RII-binding domains of ten AKAPs. The amino acid sequences of the peptides with the highest binding affinities (derived from AKAP2, AKAP5, AKAP6, AKAP7 and AKAP13) were further optimised with regard to RII binding by a combination of bioinformatics and substitution analysis of all positions. The resulting final high-affinity peptide was termed AKAP in silico (AKAP$_{IS}$, RI$\alpha$: $K_d = 0.23 \pm 0.05\ \mu M$, RII$\alpha$: $K_d = 0.45 \pm 0.07\ nM$). The ability of AKAP$_{IS}$ to disrupt PKA-anchoring was revealed by its ability to evoke a rapid reduction of GluR1 receptor currents in whole cell patch-

clamp experiments. The dual specificity AKAPs, AKAP1 and AKAP10 (D-AKAP1 and D-AKAP2), bind both RI and RII subunits of PKA (see above). Burns-Hamuro *et al.* [95] chose the PKA-anchoring domain of AKAP10 as a basis for the generation of the peptide AKB-RI (where AKB is A-kinase-binding). It binds RI$\alpha$ subunits with approximately 90-fold higher affinity ($K_d = 5.2 \pm 0.5\ nM$) than RII$\alpha$ subunits ($K_d = 456 \pm 33\ nM$) and thus preferentially disrupts AKAP-RI interactions. In addition, the peptide AKB-RII with reversed binding characteristics was developed [95]. It binds RI$\alpha$ subunits with almost 1000-fold lower affinity ($K_d = 2493 \pm 409\ nM$) than RII$\alpha$ subunits ($K_d = 2.7 \pm 0.1\ nM$) [95]. In recent studies two further R subunit-specific anchoring disrupting peptides were generated: RIAD (RI-specific) and SuperAKAP$_{IS}$ (RII-specific, see Table 1.1). Among the AKAPs binding RII subunits with high affinity is AKAP7$\delta$ (RII$\alpha$: $K_d = 31\ nM$; RII$\beta$: $K_d = 20\ nM$) [71]. Truncated versions of AKAP7$\delta$-(124-353) bind RII subunits with even higher affinity than the full-length protein [71]. This AKAP7$\delta$ emerges as an interesting candidate for the development of peptidic disruptors of PKA anchoring.

Table 1.1: Peptides as disruptors of AKAP-PKA binding. The binding affinity of previously described peptides to RI or RII subunits indicates their potency as disruptors. Displayed are the peptide names, the cognate sequences, the equilibrium dissociation constants ($K_d$) for RI or RII subunit binding and the properties of the peptides (n.s., non-selective RI and RII anchoring disruptor; n.b., no binding).

| **peptide name** | **sequence** | **$K_d$ (nM,mean ± SEM)** | | **properties** |
|---|---|---|---|---|
| | | RI | RII | |
| Ht31 | DLIEEAASRIVDAVIEQVKAAGAY | 1300±60 [94] | 4.0±1.2 [93] 2.2±0.03 [94] | n.s. |
| Ht31-P | DLIEEAASRPVDAVPEQVKAAGAY | n.b | n.b | neg. control |
| AKB (dual) | VQGNTDEAQEELAWKIAKMIVSDVMQQ | 48±4 | 2.2±0.2 | n.s. [95] |
| AKB (RII) | VQGNTDEAQEELLWKIAKMIVSDVMQQ | 2,493±409 | 2.7±0.1 | RII-specific [95] |
| AKB (RI) | FEELAWKIAKMIWSDVFQQ | 5.2±0.5 | 456±33 | RI-specific [95] |
| AKB (null) | VQGNTDEAQEELAWKIEKMIWSDVMQQ | 998±66 | > 10,000 | neg. control [95] |
| AKAP$_{IS}$ | QIEYLAKQIVDNAIQQA | 230±50 | 0.45±0.07 | n.s. [94] |
| SuperAKAP$_{IS}$ | QIEYVAKQIVDYAIHQA | n.b. | ≤0.45 | RII-specific [96] |
| RIAD | LEQYANQLADQIIKEATE | 1.0±0.2 | 1760±290 | RI-specific [97] |